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Phytochemical screening and assessment of wound healing activity of the leaves of *Anogeissus leiocarpus*

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ABSTRACT

Wound healing disorders present a serious clinical problem of medical health care in Africa and in Ghana; most of these disorders lead to complications, high morbidity and mortality rates. However, most of the synthetic drugs currently used for the treatment of wounds are not only expensive but also pose problems such as allergy and drug resistance. *Anogeissus leiocarpus* (Combretaceae (combretoideae)) is a well known plant in Ghana and because of its use in Ghanaian folk medicine as a wound-healing agent; the present study was carried out to investigate its wound healing activities. The parameters studied included rate of % wound contraction, days of complete wound healing and the antimicrobial activity of the plant extract. The crude methanol extract was also screened for Total phenolic content, total flavonoid content, antioxidant ability using ferric reducing antioxidant power (FRAP) assay and the presence of phytoconstituents. From the FRAP assay, absorbance increased with increasing concentration of plant extract indicating a significant reduction potential of the plant. Similarly, high values were recorded for the total phenolic and flavonoid contents, the secondary metabolites synthesized in plants known to possess antioxidant activities. The result of the study relative to wound healing was very interesting. There was a progressive decrease in wound area with time, indicating an efficacy of the formulations in healing the induced wounds. By the 15th day, the mixture containing 100 mg / ml of aqueous extract and 10 % w / w of powdered ointment of *A. leiocarpus* showed 100 % healing similar to the standard antibiotic (2% w/w penicillin). The plant *A. leiocarpus* topically possesses wound healing activity in a dose dependent manner and thus provides a scientific rationale for the traditional use of this plant in the management of wounds. As such could be developed into an alternative drug in wound healing.

Keywords: Wounds, *Anogeissus leiocarpus*, healing, antimicrobial activity, phenolic content

INTRODUCTION

Wound is defined as a disruption of cellular, anatomical, and functional continuity of a living tissue. Wounds are the result of injuries to the skin that disrupt the soft tissue. It may be produced by physical, chemical, thermal, microbial, or immunological insult to the tissue. Wounds represent a significant burden on the patients and health care professionals worldwide.

Wounds affect physical and mental health of millions of patients and impose significant cost on patients. Wounds are major cause of physical disabilities. Current estimates indicate that nearly 6 million people across the globe suffer from chronic wounds [1]. All wounds contain bacteria and even if the wound is healing normally, a limited amount of bacteria will be present. But if the bacteria count rises, the wound may become infected. Bacterial overload in a wound can lead to a serious infection that requires antibiotic treatment.

In the past, commercial antibiotics were successful to fight these infections however; the future effectiveness of antimicrobial therapy is somewhat in doubt. Microorganisms, especially bacteria, are becoming resistant to more and more antimicrobial agents.

A large number of plants are used by traditional medical practitioners in many countries for the treatment of wounds and burns. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. The herbal extracts and fractions effectively arrest bleeding from fresh wounds, inhibit microbial growth and accelerate wound healing [2]. These phytochemicals are not only cheap and affordable but also safe. Records have it that different parts of plants used for wound healing contain some active principles or components that are antimicrobial and nutritive in function [3].

The presence of various life-sustaining constituents in plants has urged scientist to examine plants with a view to determine potential wound healing properties [4].

Anogeissus leiocarpus is a plant that has been identified among the Ewes in the Kpando district in the Northern part of Volta region, Ghana, for wound healing. It belongs to the phylum, Tracheophyta; Order; *Myrtales* and Family: *Combretaceae* (combretoideae). It is commonly called Axle-wood tree, and in Ghana, it is referred to as hehe (Ewes, Krepi), Kojoli (Fulani), Annum (Kanuri), Ayin or Orin-odan Ainy (Yoruba), Atara (Igbo) and Kukunchi (Nupe). In this study we assess the phytoconstituents and the wound healing ability of *Anogeissus leiocarpus*. Botanicals with antioxidant or free radical-scavenging activity are believed to play a significant role in healing of wounds [5]. Hence the study also sought to determine the antioxidant property using FRAP assay and quantify both phenolic and flavonoid contents in the plant.

MATERIALS AND METHODS

Materials

The following materials were purchased from their local suppliers and used without further purification. Penicillin (Glaxo, Nigeria), citric acid, sodium hydroxide (Merck), acetone (BDH, England), concentrated HCl, ketamine chloride injection (Gracure pharmaceuticals Ltd., India), gallic acid monohydrate (Sigma Chemical company), Quercetin dehydrate 99% (Acros Organics), Folin & Ciocalteu's phenol reagent (Fischer Scientific). All other reagents were of analytical grade and were used as such.

Plant material and sample preparation

Fresh leaves of *A. leiocarpus* were collected from Kpando in the Volta Region of Ghana during the month of March-2012. The plant material was properly identified by Mr. Agyarkwa and Mr. Otoo, the Curators at the Herbarium of School of Biological Sciences, University of Cape Coast, Ghana. A voucher specimen was deposited in University of Cape Coast Herbarium.

The leaves were washed and shade dried. The dried leaves were milled using Vivekananda Madras Mill (1975, USA).

Preparation of methanol and aqueous plant extracts

A mass of 40 g weight of the powdered plant material was extracted with 40 ml of 70 % methanol. The mixture was heated for 30 minutes and the resulting liquid was filtered using filter paper (Whatman No 3, Whatman Ltd., England). Extraction was repeated five times and the filtrates were combined in one vessel. The solvent was removed on a water bath at 40 °C. The resulting dried mass was then powdered, packed into a glass vial and stored in a desiccator over silica gel until use. The same procedure was followed to obtain the aqueous extract (ALAE) with a higher temperature used to remove the solvent.

Wound healing activity

Experimental animals

Albino Wistar rats of either sex weighing 150-300 mg were used for the study. The animals were maintained under hygienic conditions and they were provided with commercial food pellets and tap water. Cleaning and sanitation work were done on alternate days. The cages were maintained clean and all experiments were conducted between the hours of 9 am to 5 pm.

Grouping of animals

The animal weights were recorded. The animal groupings were stratified according to Weights, so that the average weights of all groups were comparable. 6 groups of the animals with 5 rats each were used. GROUP 1 had rats treated with 5 % w/w of powdered plant ointment (5% ALEO), GROUP 2- rats treated with 10.0 % w/w of powdered plant ointment (10% ALEO), GROUP 3- rats treated with 30 mg/ml of aqueous extract (30mg ALAE), GROUP 4- rats treated with 100 mg/ml of aqueous extract (100mg ALAE), GROUP 5- rats treated with 2 % w/w penicillin ointment. GROUP 6- rats treated with Shea butter ointment and GROUP 7-rats left untreated.

Creation of excision wound

An excision wound model was used for studying wound healing activity. Fresh 50mg/ml ketamine chloride solution was prepared for anesthesia and a single-use syringe for injection. For the IV injection, the rat was held at its neck directly behind the ears and the tail was grasped while holding the head down. The rats were placed back into the cage to prevent agitation. The administration of the anesthetic solution prevented any movement of the animals for at least 2 hours so that the animals could be left without being restrained. Hair was removed by shaving the dorsal of all rats. A full thickness of the excision wound of approximately 490mm² and 2mm depth was created along the markings using toothed forceps and pointed scissors. The back of the anesthetized rats were shaved using the razor blade and the hair was carefully removed from the back of the animal. The anesthetized and shaved rats were placed on a paper towel. The shaved back of the animal was wiped with a sufficient amount of 70 % alcohol. The rats were held at the neck directly behind the ears and the tail of the rats was held down. The back of the skin was lifted using forceps. The skin was incised first and carefully cut using the scissors. Lifting up the skin ensured that the incision will move through the panniculus Carnosus. After completion of excision wounding, the wound was left undressed to the open environment and no local or systemic anti-microbial agents were used before the animals were transferred into cages.

Determination of microbial load on the wound

Swabs were taken from the excision wound each on day 5, 10, and 15. The collected swabs were immediately sent to the laboratory for testing. In the quantitative count study, each swab stick was added to 2 ml of peptone water. The sample was mixed thoroughly and a 5-fold serial dilution was performed. A volume of 0.1ml of each sample dilution was spread onto MacConkey and blood agar plates. They were incubated at 37°C for 24 hours. The colonies were counted and the results were recorded.

Phytochemical screening

Chemical tests were carried out on the methanolic extracts for the qualitative determination of phytochemical constituents as described by [6, 7, 8]. Below is a brief description of the methods used.

Alkaloids: The chloroform extracts were evaporated to dryness and the residues were heated with 2% HCl solution on a boiling water bath. The extracts were cooled, filtered and then treated with the Mayer's reagent. The sample was then observed for the presence of yellow precipitation or turbidity.

Flavonoids: 1.5 ml of a 50% aqueous methanol was added to 4 ml of plant extracts. The solution was warmed and magnesium turning was added. 5 to 6 drops of concentrated HCl was added to the solution and observed for red coloration.

Tannins: To 0.5 ml of extract solution, 1 ml of distilled water and 1 to 2 drops of ferric chloride solution was added to it, and observed for blue or green black coloration.

Saponins: 2 ml of distilled water was added to 2 ml of the test solution and shaken very well till frothing was observed.

Phenols: Ethyl alcohol was added to 2 ml of the test solution and few drops of ferric chloride solution and observed for coloration.

Determination of flavonoid contents

The aluminum chloride colorimetric method was used to measure the flavonoid content of all plant extracts [9]. Extract solution (0.25ml, 1mg/ml) of each plant extract was added to 1.25 ml of distilled water. Sodium nitrite solution (0.075ml, 5%) was then added to the mixture followed by incubation for 5 minutes after which 0.15ml of 10% aluminium chloride was added. The mixture was allowed to stand for 6min at room temperature before 0.5ml of 1 M sodium hydroxide was finally added and the mixture diluted with 0.275 ml distilled water. The absorbance of the reaction mixture was measured at 510 nm with a UV/VIS spectrophotometer immediately. Quercetin was used as the standard for the calibration curve. Flavonoid contents were expressed as mg quercetin equivalent (QE)/g dry weight (D.W.).

Determination of total phenolic content

Total phenol content was estimated using Folin-Ciocalteu reagent based assay as previously described [10] with little modification. To one ml of each extract (100 μ g/ml) in methanol, 5ml of Folin -Ciocalteu reagent (diluted ten-fold) and 4 ml (75 g/l) of Na₂CO₃ were added. The mixture was allowed to stand at 20°C for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. 1 ml aliquots of 20, 40, 60, 80, 100 μ g/ml methanolic gallic acid solutions were used as standard for calibration curve. All determinations were performed in triplicate. Total phenol value was obtained from the regression equation: $y = 0.00048x + 0.0055$ and expressed as mg/g gallic acid equivalent using the formula, $C = cV/M$; where C = total content of phenolic compounds in mg/g GAE, c = the concentration of gallic acid (mg/ml) established from the calibration curve, V = volume of extract (0.5ml) and m = the weight of pure plant methanolic extract (0.052g) (diluted ten times).

Ferric Reducing Antioxidant Power Assay (FRAP)

The reducing antioxidant power of plant methanolic extracts was determined by the method of Oyaizu [11]. Different concentrations of plant extracts (250 – 1000 ppm) in 1 ml of distilled water were mixed with phosphate buffer (3.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer (T 70 UV-VIS Spectrometer, PG Instruments Ltd). Increased absorbance of the reaction mixture indicates increase in reducing power. Ascorbic acid was used as standard.

Statistical Analysis

All the experiments were performed in triplicate and the results were expressed as mean \pm SD (standard deviation). Statistical analysis was performed using SPSS 13.0 and Excel 2003. The difference was considered significant at $p < 0.05$.

RESULTS

The flavonoid content of the extracts in terms of quercetin equivalent (the standard curve equation: $y = 0.0092x + 0.0249$, $r^2 = 0.9854$) was 330.7 ± 29 mg g⁻¹ for the methanol extract (Table 1). Table 1 also shows the contents of total phenols that were measured by Folin- Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: $y = 0.00048x + 0.0055$, $r^2 = 0.9991$). The total phenol content was 1294.8 ± 3.0 mg g⁻¹ in the methanol.

Table 1: Quantification of Phenolic and Flavonoid contents in the methanol extract

Extract	Phenolic Content (mg of GAE/g of extract)	Flavonoid content (mg of QE/g of extract).
Methanol	1294.8 ± 3.0	330.72 ± 29

From the FRAP assay (fig. 1), absorbance increased with increasing concentration of plant extract. This signified the consistent reduction of Fe³⁺ to Fe²⁺ indicating a strong reduction potential of the plant; a potential much higher than that of the standard antioxidant.

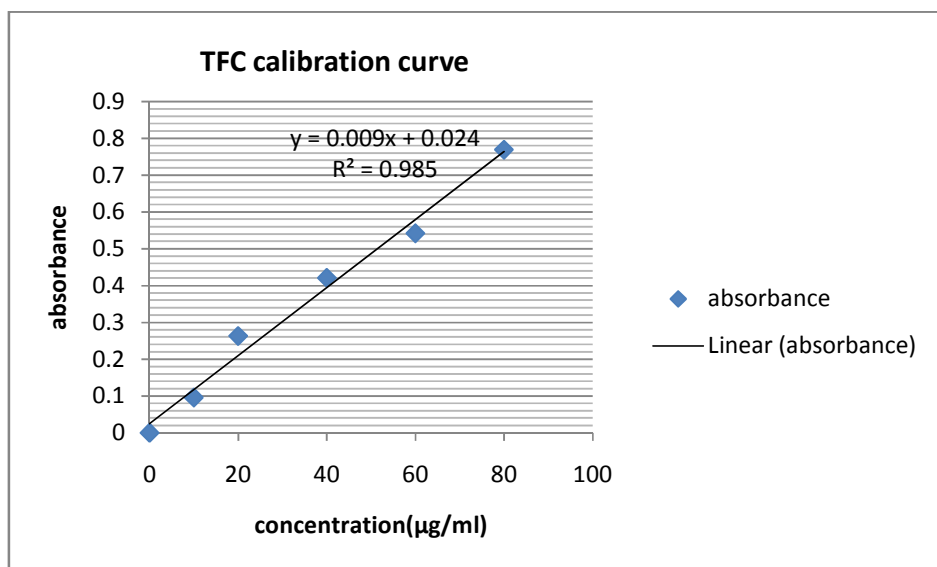
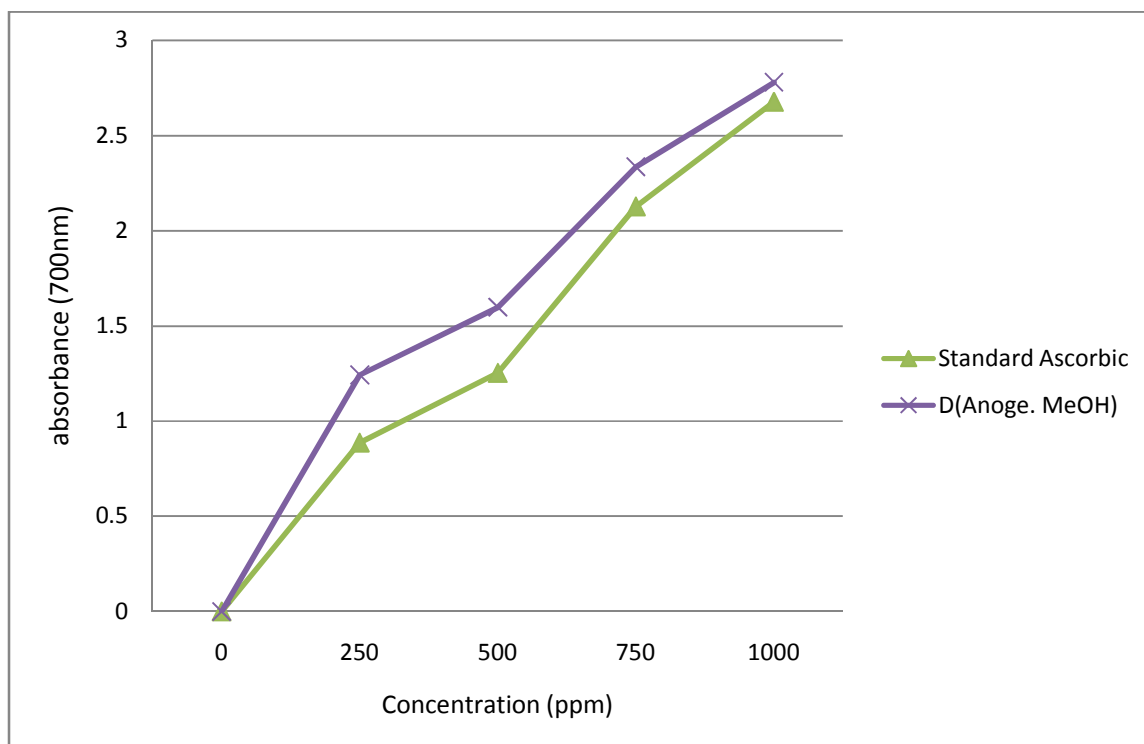


Figure 2: Calibration curve for the determination of Total Phenolic Content

Figure 1: Ferric reducing power of *A. leiocarpus* methanol extract compared with Ascorbic acid as standard

The results from the study on excision wound revealed a wound healing activity induced by the leaf extract of different formulations (ALAE 30 mg / ml, ALAE 100 mg / ml, ALEO 5 % w / w, ALEO 10 % w /w) treated groups, untreated groups (control), shea butter and 2 % penicillin ointment (standard drug) of animals. The mean percentage closure of wound area was calculated on the 3rd, 6th, 9th, 12th and 15th post wounding days as shown in table 3. Table 2 shows the result of the preliminary phytochemical screening on both the aqueous and methanol leaf extracts of the plant. The result revealed the presence of many major phytoconstituents in the plant.

Table 2: Preliminary phytochemical study of *Anogeissus leiocarpus* Leaves

Phytoconstituent	Aqueous Extract	Methanol Extract
Alkaloid	+	+
Saponins	+	+
Tannins	+	+
Anthraquinones	+	+
Flavonoid	+	+
Terpenoids	+	+
Steroids	-	+
Cardiac glycosides	-	+
Phenol compounds	+	+
Acid compounds	-	-

+ indicates presence, - indicates absence

The microbial load on the excision wounds were also monitored throughout the study period to estimate the presence of microbes that might be present to cause wound infection. The result is shown in fig. 3.

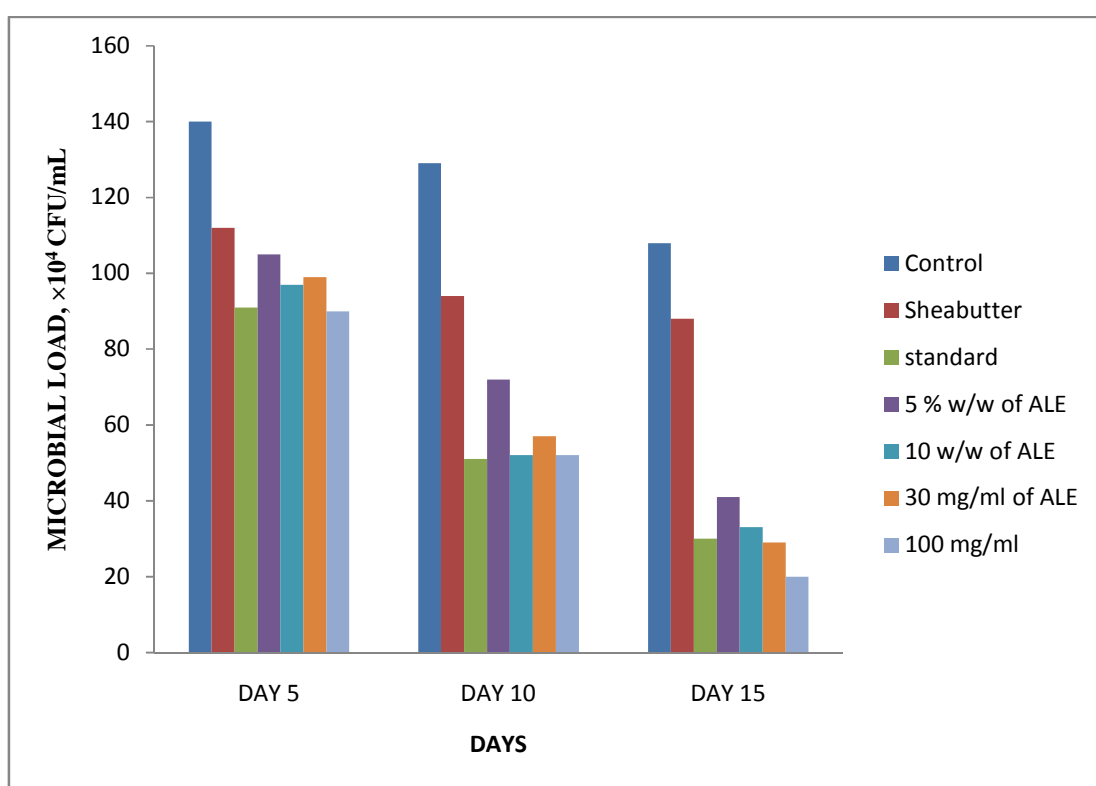


Figure 3. Comparison of the microbial load of the various rat groups that were administered with different treatments
Each bar indicates microbial load of different treated wound on the given days

Table 3: Effect of *A. leiocarpus* leave extract on excisional wound in terms of percentage wound closure

Days Treatments	DAY 0	DAY 3	DAY 6	DAY 9	DAY 12	DAY 15
Control (Untreated)	2.63 ± 0.05 (0.00 %)	1.83 ± 0.03 (27.94 %)	1.77 ± 0.08 (28.45 %)	1.29 ± 0.11 (50.88 %)	0.79 ± 0.11 (69.85 %)	0.48 ± 0.06 (81.76 %)
Shea butter treatment	2.44 ± 0.10 (0.00 %)	2.19 ± 0.10 (10.00 %)	1.83 ± 0.12 (25.07 %)	1.05 ± 0.13 (56.11 %)	0.65 ± 0.04 (72.85 %)	0.14 ± 0.06 (94.52 %)
2 % w / w penicillin ointment	2.59 ± 0.03 (0.00 %)	1.77 ± 0.06*	1.41 ± 0.08*	0.82 ± 0.12*	0.69 ± 0.09 (75.75 %)	0 (100.00 %)*
5 % w / w of drug dose	2.52 ± 0.07 (0.00 %)	1.96 ± 0.15 (22.33 %)	1.66 ± 0.07 (34.16 %)	0.91 ± 0.11* (64.30 %)	0.59 ± 0.05 (76.49 %)	0.13 ± 0.08* (95.06 %)
10 % w / w of drug dose	2.58 ± 0.12 (0.00 %)	1.97 ± 0.21 (24.00 %)	1.65 ± 0.13 (35.38 %)	0.91 ± 0.09* (64.59 %)	0.57 ± 0.07 (77.78 %)	0* (100.00 %)
30 mg / ml of aqueous extract	2.54 ± 0.05 (0.00 %)	1.72 ± 0.10 (31.46 %)	1.51 ± 0.06 (40.70 %)	1.04 ± 0.07* (62.80 %)	0.55 ± 0.05 (78.54%)	0.17 ± 0.10* (93.63 %)
100 mg / ml of aqueous extract	2.58 ± 0.08 (0.00 %)	1.74 ± 0.06* (32.44 %)	1.41 ± 0.04* (45.16 %)	0.93 ± 0.06* (63.90 %)	0.51 ± 0.06 (80.21 %)	0* (100.00 %)

Values are mean ± SEM. Numbers in parenthesis indicates percentage wound closure. * Significantly different from the control at $P < 0.05$, $n = 5$

DISCUSSION

The therapeutic benefit of medicinal plants is often attributed to their antioxidant property [4]. Antioxidants play very important roles in coetaneous tissue repair as they significantly prevent tissue damage that stimulates wound healing process. It has been mentioned that the antioxidant activity of plants might be due to their phenolic compounds [12]. Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action [13]. Flavonoids are the most important natural phenolic compounds and one of the most diverse and widespread groups of natural compounds [9]. Some evidences suggest that the biological actions of these compounds are related to their antioxidant activity [14].

The result recorded very high values for total phenolic and flavonoid contents. The high values recorded for the extract of the plant showed that *A. leiocarpus* may be a good source of antioxidant activity and this agreed vividly with the results from the FRAP assay. The FRAP value increased with increase in concentration of the extract. Substances that increase FRAP value and percentage antioxidant activity in DPPH spectrophotometric assay is assumed to have antioxidant activity [15]. It is therefore not out of place to state that *A. leiocarpus* has demonstrated good antioxidant activity from the results and it can be suggested that the plant may be useful in maintaining health and preventing degenerative diseases such as cancer, diabetes, coronary heart disease that are exacerbated by the generation of reactive oxygen species (ROS) in the body.

Similarly, treatment of the excision wounds with the different formulations of the plants gave good indications of the wound healing potency of the plant comparable to the standard drug. It was observed that the wound contracting ability of the plant and the 2 % penicillin ointment were significantly greater than that of the control. The aqueous extracts and the ointment formulation of the plant on all treated groups showed significant wound healing from the 3rd day onwards, which was comparable to that of the standard drug on the treated group of animals without any significant difference. The percentage wound contraction was much more with the 10 mg / ml ALAE and the 10 % ALEO (higher doses) than the 5 mg / ml ALAE and the 10w / w ALEO (low doses). The wound closure time was also lesser with the higher dosage treated animals as they showed lesser days of complete wound healing (15 days) than the animals treated with lower doses (17days). This showed that the plant extract was dose dependent with high doses exhibiting greater wound healing activity than lower doses.

Microbial load determination on the various treated wounds showed a significant reduction on day 5, 10 and 15 with the standard treated and the various plants extract treated groups been significant more than the control. Again reduction in microbial load was dose dependent as higher doses significantly reduced microbial load than lower doses as observed in figure 3.

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contraction is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase,

the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue.

The preliminary phytochemical analysis of aqueous and methanol leaf extract of *A. leiocarpus* revealed the presence of flavonoids, terpenoids, tannins, alkaloids, cardiac glycosides, saponins, steroids, anthraquinones and phenol compounds. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity [16]. Flavonoids, and terpenoids are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialization [17, 18]. This is very important as researchers proved that the control of microbial infection is necessary for better wound healing and its management.

Tannins, the main components of many plant extracts, act as free radical scavengers. Researches into the role of antioxidants from plant extracts in wound healing have been widely published. This gives credence to the fact that free radical scavenging action of plants as well as their antioxidant properties enhances wound healing [19]. Antioxidants are known to play very important roles in coetaneous tissue repair as they significantly prevent tissue damage that stimulates wound healing process [20]. The result indicated larger proportions of these phytoconstituents as well as high antioxidant activity in *A. leiocarpus*. Thus, the wound-healing activity of *A. leiocarpus* may be attributed to the presence of these phytoconstituents, which may be either due to their individual or synergetic effect that hastens the process of wound healing and their antioxidant activity.

CONCLUSION

In conclusion, the formulations from the extract of the plant *Anogeissus leiocarpus* topically posses wound healing activity and thus provides a scientific rationale for the traditional use of this plant in the management of wounds. As such could be developed into an alternative drug in wound healing.

Further studies are needed to find out the mechanism of these biological effects and also the active constituents responsible for these effects.

REFERENCES

- [1] Kumar B, Vijayakumar M, Govindarajan R, Pushpangadan P, *J. Ethnopharmacol*, **2007**, 114: 103-113.
- [2] Okoli CO, Akah PA, Okoli AS, *BMC Complem. and Altern. Med*, **2007**, 7, article 24.
- [3] Dalazen P, Molon A, Biavatti MW, Kreuger MRO, *Brazilia J. of Pharmacog*, **2005**, 15: 82-87
- [4] Nayak S, Nalabothu P, Sandiford S, Bhogadi V, Adogwa A, *BMC Complem. and Altern. Med*, **2006**, 5: 6–12.
- [5] Kamath JV, Rana AC, Chowdhury AR, *Phytothe. Res*, **2003**, 17: 970–972.
- [6] J.B Harborne JB, *Phytochemical Methods: A guide to Modern Techniques of Plant Analysis*, Chapman and Hall, **1998**.
- [7] Trease EG, Evans CW, *Laboratory Methods in Antimicrobial Chemotherapy*, Churchill Livingstone, **1978**.
- [8] Sofowora A, *Medicinal Plants and Traditional Medicines in Africa*, John Wiley & Sons, NewYork, **1993** pp 6-65.
- [9] Nguyen Q, Eun J, *J. of Med. Plants Res*, **2011**, 5(13): 2798-2811
- [10] McDonald S, Prenzler PD, Antolovich M, Robards K, *Food Chem*, **2001**, 73: 73-84.
- [11] Oyaizu M, *Japan J. Nutr.*, **1986**, 44: 307-315
- [12] Handa SS, Khanuja SPS, Longo G, Rakesh DD, *Extraction Technologies for Medicinal and Aromatic Plants*, ICS-UNIDO, Trieste, **2008**, pp. 21–52.
- [13] Shetty S, Udupa S, Udupa L, *Evidence-Based Complem. and Alternat. Med.*, **2008**, 5(1):95–101.
- [14] Nayak SB, Kanhai J, Milne DM, Pereira LP, Swanston WH, *Evidence-Based Complem. and Alternat. Med*, **2011**, 2011:6.
- [15] Ramdas P, Seema M, *Int. J. Pharm. Pharm. Sci*. **2010**, 2 (4), 68–73.
- [16] Olukemi OA, Sofidiya MO, Aderonke TS, Ajose I, Onalo M, Shauib B, *Annals of Biol. Res*, **2012**, 3(4): 1683-1789.
- [17] Tsuchiya H, Sato M, Miyazaki T, Fujiwara S, Tanigaki S, Ohyama M, *J. Ethnopharmacol*, **1996**, 50:27–34
- [18] Scortichini M, Pia Rossi M, *J. Bacteriol*, **1991**, 71:109–12.
- [19] Süntar I, Küpeli AE, Nahar L, Satyajit D, Sarker SD, *Free Radicals and Antioxidants*, **2012**, 2, 2: 1-7.
- [20] Barku VYA, Opoku-Boahen Y, Owusu-Ansah E, Mensah EF, *Asian J. Plant Sci. Res*, **2013**, 3(1):69-74