PRELIMINARY PHYTOCHEMICAL SCREENING, ANTIMICROBIAL EXAMINATION AND WOUND HEALING POTENTIAL OF THE ROOT EXTRACT OF *AMARANTHUS SPINOSUS*.

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Abstract

Amaranthus spinosus is traditionally acclaimed to be effective in treating infectious wounds in man and animals. It is also used medicinally in the treatment of menstrual disorders in women, eczema, burns, earache, haemorroids, lactogogue and a specific treatment for colic. In spite of all these extensive medicinal uses of *A. Spinosus*, not much scientific studies have been done, in particular on its wound healing effects and phytochemical composition. A preliminary phytochemical screening, antimicrobial properties and wound healing potential of the root extract were determined. Additionally, the unsaponifiable matter and the total fatty acid content of the seed oil were determined. Exhaustive soxhlet extraction using petroleum-ether (40-60 ^{0}C) yielded 4.45g of fixed oil which was found to contain 2.82g (73.06%) unsaponifiable matter and 1.04g (26.94%) total fatty acids. Preliminary phytochemical screening on the unsaponifiable matter gave positive results for steroids, terpenoids and flavonoids whiles both methanol and aqueous extract were additionally positive for cyanogenic glycosides, tannins, reducing sugar phenolics and saponins. Column chromatography on the unsaponified matter using MeOH/$CHCl_3$ (1:2) and 100% MeOH followed by TLC (Benzene-Ethyl acetate (9:1) yielded four and three possible components respectively with distinct colours when viewed under UV light. Anti-microbial sensitivity effect of the fixed oil against *Escherichia coli* and *Staphylococcus aureus* produced minimal anti-microbial activity compared to the reference anti-biotics (Chloroamphenicol and Tetracycline). The results from excision wound model showed that the *A. spinosus* extract has wound healing capacity, as evident from better wound closure and improved tissue regeneration at the wound sites. The effect of treatment on microbial load on wounds saw a decrease in the bacteria on the surface of the wounds corresponding to the activity of the extracts and the other preparations.

Introduction

Antimicrobials of plant origin have enormous therapeutic potential in the treatment of infectious diseases (Iwalewa et. al, 2007). They are effective as well as have the advantage of mitigating many of the side effects that are often associated with synthetic antimicrobials (Mcgaw, et. al, 2008). Different extracts

from traditional medicinal plants have been tested to identify the sources of their therapeutic effects. As a result some natural products have been approved as new antibacterial drugs, but there is still an urgent need to identify novel substances with activity against pathogens with high resistance (Cragg et al., 1997; Recio, 1989). *A. spinosus* contains lot of substances having medicinal value, which are yet to be explored. The plant has several active constituents like alkaloids, flavonoids, glycosides, phenolic acids, steroids, amino acids, terpenoids, lipids, saponins, betalains, ß-sitosterol, stigmasterol, linoleic acid, rutin, catechuic tannins and carotenoids. It also contains amaranthoside, a lignin glycoside, amaricin, a coumaroyl adenosine along with stigmasterol glycoside, betaine such as glycinebetaine and trigonelline (Azhar-ul-Haq *et al.*, 2006; Blunden *et al.*, 1999). Additionally, Azhar-ul-Haq et al., (2004) isolated for the first time α- xylofuranosyl uracil, β-D-ribofuranosyl adenine and β-sitosterol glucoside and Spinoside, a new coumaroyl flavone glycoside from the n-butanol fraction of methanolic extract of the whole plant of *Amaranthus spinosus*. It has also been reported that *A. spinosus* and some other medicinal plants are used in the Kpando Municipality as folkloric medicine in the treatment of wound and wound infections (Barku et al., 2013).

The medicinal value of *A. spinosus* has made it a very important plant that needs thorough investigation. Many investigators have carried out experiments on the different parts of the plant to ascertain the medicinal value of this plant. For example, a study conducted by Ibewuike *et al.,* 1997 on the methanol extract of the leaves of *A. spinosus* (25-100 mg / kg) in different animal models significantly inhibited carrageenan-induced rat paw oedema and produced significant inhibition of acetic acid-induced increase in vascular permeability. This indicated the anti-inflammatory activity of the extract. *A. spinosus* extract also exhibited a highly specific prostaglandin synthesis inhibitory activity *in vitro* in an anti-inflammatory model test system, indicating its anti-inflammatory properties (Olufemi et al., 2003*)*. Similar experiment conducted by Chaudhary et al., 2012, on the crude methanolic extract of *A. spinosus* indicated that the plant possesses laxative, bronchodilator and spamolytic activities. Nevertheless, there are still more useful properties yet to be tapped on the plant, especially its wound healing effect and phytochemical properties since it has been posited that the therapeutic effect of medicinal plants originate from their phytochemical composition. Despite the wide medicinal uses of *A. spinosus,* no scientific data is available with respect to its effectiveness in wound healing. Similarly, scanty information can be obtained on the properties of the fixed oil in the roots of *A. spinosus*. This preliminary study on the root was therefore undertaken to explore wound healing properties of *A. spinosus* and to consider further the medicinal value and the phytochemical composition of the fixed oil.

Experimental

REAGENTS

Hexane, pet ether, Hydrochloric acid, sodium hydroxide, anhydrous sodium sulphate, diethyl ether, chloroform, methanol, ethanol, ethyl acetate were obtained from the Central Store, Department of Chemistry, UCC. Nutrient agar, Mueller Hinton agar was obtained from the School of Biological Sciences Central Store.

Plant Collection, Identification and Authentication

The plant was identified and authenticated at the herbarium unit of the School of Biological Sciences, University of Cape Coast, Ghana, where a voucher specimen **(CCG / 5165)** was deposited.
The roots of *A. spinosus* were collected from Ejisu - Onwe, in the Ashanti Region of Ghana in January, 2013. The roots were washed and sun-dried for three weeks. The dried roots were chopped into smaller pieces, milled into fine powder using a Mill (Amonda Grinding Mill, mode 2, India) and stored in airtight bottles.

Extraction of Petroleum-ether extract

Five hundred and twenty eight gram of pulverized plant material was extracted with 1.5 litres of petroleum-ether (60-80^{0}C) for 16 hours. The solvent was distilled off under reduced pressure to yield deep brownish yellow coloured fatty oil (4.45g) which was found to have the characteristics as described in Table 1.

Preparation of Methanol Extract

A mass of 100 g of the powdered sample was cold macerated with 700 ml of 70 % methanol for one week. The cold macerate was filtered (whatman filter paper, No 1) and the 70 % methanol retrieved from the filtrate using a rotary evaporator (EYELLA CCA – 1111, Japan) maintained at 50 °C. The filtrate extract was frozen in a dry ice–acetone bath and then freeze-dried (Hecto Power Dry LL30000, Manufactured by Jouan Nordic, Denmark). When the extract was completely dried, it was collected and placed in a moisture-free package, sealed and kept in desiccator until use. The dried extract was weighed to get a percentage yield of 14.5 %.

Preparation of Aqueous Extract

A mass of 100 g of the powdered sample was boiled in 1 litre distilled water for 45 minutes. The mixture was allowed to cool and filtered on a Buchner funnel using filter paper (Whatman filter paper, No 1). The filtrate was frozen in a dry ice–acetone bath and then freeze-dried. The dried extract (10.0%) was placed in a moisture-free package, sealed and kept in a desiccator until use.

Extraction of unsaponifiable Matter and Total Fatty Acid

A portion of the oil obtained from the petroleum-ether extract was weighed (3.86g) and dissolved in ethanolic potassium hydroxide (20 ml 96% ethanol and 0.8g/ml KOH). The content was refluxed for 1 hour. The hot solution was cooled to room temperature and distilled water (40ml) was added. The solution was extracted with diethyl ether (3 x 10ml). The ether extract containing the unsaponified matter was separated from the saponified material of the soap solution in a separatory funnel. The ether extract was washed with 0.1M NaOH (3 x 1ml), followed by two drops of concentrated HCl, distilled water (3 x 1ml) and dried over anhydrous sodium sulphate. It was evaporated under pressure to yield the unsaponifiable matter (2.82g, 73.06%).

Concentrated HCl was added to the aqueous soap solution until it was acidic to litmus. It was extracted with diethyl ether (3 x 10ml). The ether extract was washed with sodium chloride solution (3 x 5ml), followed by distilled water (3 x 5ml) and dried over anhydrous sodium sulphate. It was evaporated under pressure to yield a white solid of the total fatty acids mixture (1.04g, 26.94%).

Phytochemical Screening

Phytochemical screening was carried out on the unsaponified matter, methanol extract and the aqueous extract using the standard methods (Sofowora, 1993, Trease and Evans, 1992).

Column and Tlc Analysis on unsaponifiable matter

A chloroform bed of silica gel was prepared and 2.45g of unsaponifiable matter was eluted in a column chromatography with Petroleum ether (100%), chloroform-methanol (2:1) and methanol 100% as the elution solvents. The various fractions obtained were spotted on a TLC plate and developed with benzene-ethyl acetate (9:1).The plates were observed under UV lamp and when exposed to iodine vapour.

Excision wound model

Healthy male Sprague Dewley Albino rats of weights 234 - 389 g obtained from the breeding unit of Centre for Scientific & Research into Plant Medicine (CSRPM) at Mampong-Akwapim in Ghana were used for the study. They were maintained under normal ambient conditions of temperature, relative humidity and day/night cycle. The rats were housed in sanitized metal cages (60 × 38 × 25 cm) with a base dressing of sawdust as bedding. The rats had free access to standard pellet diet (GAFCO, Tema, Ghana) and water.

The animals were anesthetized into a state of anesthesia one after the other by intramuscular injection of 50 mg / kg ketamine chloride (Gracure Pharmaceuticals Ltd, batch / lot K-022, India). The skin area was shaved using surgical blade (size 11, Shanghai Hess. Imp. & Exp. Co. Ltd, China). The skin of the shaved area was excised to full thickness to obtained a circular wound of area 314.2 mm^2 using a surgical blade (size 11, Shanghai Hess. Imp. & Exp. Co. Ltd, China), pair of scissors (8 mm × 1 mm × 2 mm, Shanghai Hess. Imp. & Exp. Co. Ltd, China) and a pair of forceps (18 mm × 1 mm, Shanghai Hess. Imp. & Exp. Co. Ltd, China), on the depilated ethanol sterilized lateral region thus specifically sacral region of the rats. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. Excision wounds were done in the mornings. Each rat was used only once. The animals were grouped into eight (8) with four (4) rats per group. The various groups were treated as follows: Group 1 (Penicillin using 1 cm spatula end), Group 2 (Drez using 1 cm spatula end), Group 3 (5 % powdered plant extract in Shea butter using 1 cm spatula end), Group 4 (10 % powdered plant extract in Shea butter using 1 cm spatula end), Group 5 (50 mg / ml of aqueous extract using 1 ml syringe), Group 6 (100 mg / ml of aqueous extract using 1 ml syringe), Group 7 (Shea butter only using 1 cm spatula end) and Group 8 (untreated). The rats were treated once daily from day zero after wound creation till complete epithelialization was achieved. The rate of wound closure was determined by measuring

diameter of wound while the animals were under anesthesia on the 0, 3rd, 6th, 9th, 12th, and 15th day until complete wound healing was achieved. The percentage wound closure was calculated for each group using the measured diameter of the wounds.

Determination of Effect of Treatments on Microbial Load of Wounds

Normal saline was used to clean the harden surface of the wound. Swabs were then taken from the excision wounds on days 4, 8, and 12 using sterile swab stick. Sterile peptone water was inoculated with the swab sample and incubated for 20 minutes at 37^{o}C. Test tubes were arranged in a rack and filled each with 9 ml of 0.85 % physiological saline. Bacterial suspension of 1ml was pipette after the 20 minutes of incubation into the first test tube and was mixed thoroughly. A volume of 1ml of diluted suspension was transferred from the first tube into the second tube and was mixed thoroughly. The dilution was continued in this fashion to the last tube to serially dilute the original suspension and 1ml of diluted suspension was discarded from the last tube. A volume of 0.1ml of the bacteria suspension was transferred from each tube onto the center of plate count agar plates and spread evenly over the surface with a sterile, L-shaped glass rod. The glass rod was sterilized by dipping in 70 % isopropyl alcohol and was then flamed. The plates were incubated at 37^{o}C for 18-24 hours and colony counting was performed using a Quebec colony counter (Reichert Instruments, USA). The plate that gave a total colony forming units (CFU) of 30-300 was selected and used to estimate number of bacteria per ml.

3.8. Bacteria Count

To calculate the number of bacteria per ml of diluted sample the following equation was used:

$$\text{Bacteria per ml} = \frac{\text{Number of CFU}}{\text{Volume plated (ml) x total dilution used}}$$

Total dilution = 10^{-3}

Volume plated = 0.1ml

Antimicrobial Assay

The oil was used for bacteria sensitivity test on the following microbes; *Escherichia coli* and *Staphylococcus aureus* using Kirby-Bauer disc diffusion method.

Preparation of Nutrient Agar and Subculturing of Test Organism

Nutrient agar (0.67g) was dissolved in 24 ml of deionised water and boil for 10 minutes in a microwave. This solution was then autoclaved at 121°C and cooled to 47°C before pouring it into Petri dishes which have been autoclaved for 45 minutes and disinfected with absolute ethanol and the media surface dried in a laminar hood for 15minutes.The various test organisms thus *Esherichia coli* and *staphylococcus aureus* were sub cultured on the nutrient media in their appropriate petri dish for 18 hours in an incubator.

Preparation of Mueller-Hinton Agar

About 1.9g of nutrient media (Mueller-Hinton) was dispersed in 50ml of deionised water. This solution was soaked for 10 minutes, swirled to mix and sterilized by autoclaving for 15 minutes at 121°C. it was cooled to 47°C and mixed before pouring it into petri dishes which have been autoclaved (heat sterilization under pressure of 1 bar) for 45 minutes and disinfected with absolute ethanol and the media (Mueller-Hinton) surface dried.

Preparation of Inoculum

The incubated media containing the test organisms were obtained for the inoculums. Five or four isolated colonies of the two test organism were suspended in 2ml of sterile saline solution respectively and vortex to create a smooth suspension.

Sensitivity Test

The organism to be tested was inoculated in saline solution (an isotonic solution of sodium chloride and distilled water). The Mueller- Hinton agar medium was also inoculated with the inoculated saline solution by three dimensional streaking. Agar diffusion wells were created on the media surface. The disc were impregnated with the varied volume of the crude extracts and allowed to dry before they were placed unto the inoculated plates for incubation. The plates inoculated with test organisms were incubated overnight at a temperature of 35-36°C. The diameters of the inhibition zone were measured in millimeters using a ruler. Each antimicrobial assay was performed in triplicates and the values recorded.

Statistical analysis

The resulting data were analyzed statistically and interpreted by using Statistical Package for Social sciences (SPSS) version 16.0. One way analysis of variance (ANOVA) was used to determine the significance of the differences in the means for the various groups. A confidence interval of 95 % (α = 0.05) was chosen. The results were considered statistically significant at Probability value ($P < 0.05$).

Results and discussion

Results on the yield of the oil are presented in table 1. The oil has a yellowish brownish colour, more viscous and solid at room temperature, it has very appealing odour. Soxhlet extraction of 528g of dried root of *Amaranthus* spinosus yielded 4.55g (0.84%) of fixed oil; this supports the evidence that the plant contains some fixed amount of oil although the yield is of low value for any economic value.

3.86g of the *Amaranthus spinosus* oil yielded 2.82g (73.06%) of unsaponifiable matter and 1.04g (26.94%) of total fatty acids respectively. The high yield of unsaponifiable matter suggests that the oil contains a lot of wax materials and hence not good for soap making.

Table 1: Physical characteristics of the Fatty oil extracted from petroleum-ether.

Variables	Characteristics
Yield	0.84%
Unsaponifiable matter	73.06%
Total fatty acid	26.94%
Colour	Deep brownish yellow
Solubility	Sparingly soluble in ethanol
Stain	leave a permanent stain on filter paper
State	solid at room temperature, non volatile

Phytochemical screening

Preliminary phytochemical screening of the unsaponifiable matter for Flavonoids, Terpenoids and Steroids showed positive result as shown in Table 2. Similarly, both the aqueous and methanolic extracts gave positive results for tannins, saponins, reducing sugars, phenolics and cyanogenic glycosides in addition to the above mentioned constituents. The unsaponifiable matter was separated into two main fractions on column using silica gel as the stationary phase.

Table 2: Results of phytoconstituents in both methanolic and aqueous extracts of *A. spinosus*

Phytocompounds	Aqueous Extract	Methanol Extract	Unsaponifiable extract
Saponin	+	+	
Reducing sugar	+	+	
Phenolics	+	+	
Tannins	+	+	
Flavonoids	+	+	+
Terpenoids & Steroids	+	+	+ +
Cyanogenic glycosides	+	+	

+ indicates presence

Fraction one yielded a deep yellow colour substance from $CHCl_3$: MeOH (2:1 solvent system). Fraction two yielded a slightly yellow colour substance from MeOH as the eluting solvent.

Thin layer chromatography technique was used to give a rough idea of the number of components present in the isolated fractions. Result from TLC analysis for the different fractions is as shown in Table 3.

Chloroform: Methanol fraction showed four different spots with their characteristic colours visible under UV lamp while three distinct spots with their characteristic colours were visible under UV lamp for the Methanol fraction. The findings suggest four possible components for the less polar fraction (Chloroform: Methanol) and three components for the more polar fraction (Methanol). The results thus confirm the presence of some important phytoconstituents in the fixed oil and the other extracts of the plant that may contribute to the rich medicinal potentials of this plant.

Table 3: TLC observation on the two fractions isolated from the coloumn **(**Mobile Phase – Benzene: Ethyl acetate (9:1)

Spots	**$CHCl_3$: MeOH (2:1)**		**MeOH**	
	Colour	R_f (cm)	Colour	R_f (cm)
1	Light green spot	0.19	Light green	0.27
2	Cream spot	0.35	Pale blue	0.48
3	Pale blue spot	0.54	Light yellow	0.62
4	Light yellow spot	0.73		

The oil was tested for antimicrobial activity against one gram positive and one gram negative bacteria using paper disc plate method of Cheesbrough (2006). In the same way controls were ran with solutions of Chloroamphenicol and tetracycline. The results recorded in Table 4 are the averages of the three replicates. The oil has shown a feeble activity (comparable to chloroamphenicol and tetracycline). However, it is an ample indication that the oil has some appreciable level of antimicrobial activity. This explains further the wound healing potential of the plant.

Table 4: Antibacteria activity of fixed oil from the roots *Amaranthus spinosus* against bacteria strain

	zone of inhibition (mm)		
Test organism	Extract	Chloroamphenicol	Tetracycline
***E.coli* (-)**	11.83 ± 0.62	26.00 ± 2.00	-
***S.aureus* (+)**	12.75 ± 0.54	-	25.50 ± 1.50

Wound healing activity

All the prepared formulations: PFE (5 % powder in Shea butter), PTE (10 % powder in Shea butter), AFE (50 mg / ml aqueous extract) and ATE (100 mg / ml aqueous extract) produced a significant decrease in period of epithelization. The mean percentage wound closure was calculated on the 0, 3rd, 6th, 9th, 12th, and 15th post-wounding days as shown in Table 5. A significant difference in wound closure was observed between the groups from day 0 onwards; in later days (day 9), the

rate of wound closure in the treated group was as faster as that in the control group as shown in Table 5.

Table 5: Effect of topical application of *A. spinosus* extracts on excision wound model recorded in % wound contraction

Treatment	DAY0	DAY3	DAY6	DAY9	DAY12	DAY 15
Penicillin (Standard)	19.50 ± 0.20 (0.00%)	12.67 ± 1.66 (35.04%)	9.33 ± 0.82 (52.14%)	4.42 ± 0.57 (77.35%)	3.00 ± 0.55 (84.62%)	0.50 ± 1.00 (97.44%)
Drez (Standard)	19.00 ± 0.47 (0.00%)	12.67 ± 1.06 (33.33%)	8.67 ± 1.25 (54.39%)	3.75 ± 0.92 (80.26%)	2.17 ± 1.55 (88.60%)	0.67 ± 1.34 (96.49%)
PFE	19.00 ± 0.69 (0.00%)	14.00 ± 1.36* (26.64%)	8.17 ± 1.55 (57.21%)	3.25 ± 0.42 (82.97%)	1.08 ± 0.74 (94.32%)	0.00 ± 0.00 (100%)
PTE	19.08 ± 0.57 (0.00%)	13.09 ± 1.75 (31.44%)	8.42 ± 0.96 (55.90%)	4.09 ± 0.96 (78.60%)	2.92 ± 0.69 (84.72%)	0.83 ± 1.67 (95.63%)
AFE	19.25 ± 0.42 (0.00%)	9.50 ± 1.00* (50.65%)	7.92 ± 1.83 (58.87%)	3.17 ± 0.88 (83.55%)	1.83 ± 1.40 (90.48%)	0.00 ± 0.00 (100%)
ATE	19.17 ± 0.33 (0.00%)	10.50 ± 0.80 (45.22%)	7.92 ± 0.69 (58.70%)	3.25 ± 1.03 (83.04%)	1.75 ± 1.42 (90.87%)	0.00 ± 0.00 (100%)
Shea Butter Only	19.25 ± 0.32 (0.00%)	13.75 ± 3.18 (28.57%)	9.34 ± 1.46 (51.52%)	4.09 ± 0.63 (78.79%)	1.67 ± 1.19 (91.34%)	0.00 ± 0.00 (100%)
Control (Untreated)	19.59 ± 0.17 (0.00%)	10.59 ± 1.50 (45.96%)	8.17 ± 0.43 (58.30%)	2.83 ± 0.58 (85.53%)	1.50 ± 1.37 (92.34%)	0.00 ± 0.00 (100%)

Values are mean ± SD (n = 4). Numbers in parenthesis indicate percentage wound closure. * Significant at $P < 0.05$ compared with standard drugs. PFE – 5 % *A. spinosus* powder in Shea Butter. PTE – 10 % *A. spinosus* powder in Shea Butter. AFE – 50 mg / ml Aqueous *A. spinosus* extract. ATE – 100 mg / ml Aqueous *A. spinosus* extract.

Determination of effect of treatments on microbial load of wounds

The topical application of drugs is an efficient therapy of destroying microbial populations because the availability of the drug at the infected wound site leads to enhanced wound healing activity. The virulence capacity of microorganisms and host immune response are important factors that can cause massive

damage during infection. The total bacterial counts from granulation tissue on different days are shown in Fig. 1. The estimation of microbial load on the surface of the wounds had a similar trend just like the wound healing ability. The application of plant extract resulted in diminishing total bacterial counts in the infected wound from days 5, 10 and 15. There was significant decrease in the number of microbes in the wounds with plant extract treatments and was also dose dependent. Earlier research had revealed that alkaloids, tannins, Cyanogenic glycosides and saponins have antimicrobial property which contributes to the medicinal property of *A. spinosus* (Oboh et al., 2009). Therefore the order of antimicrobial activity observed is in line with the phytoconstituents present in the plant.

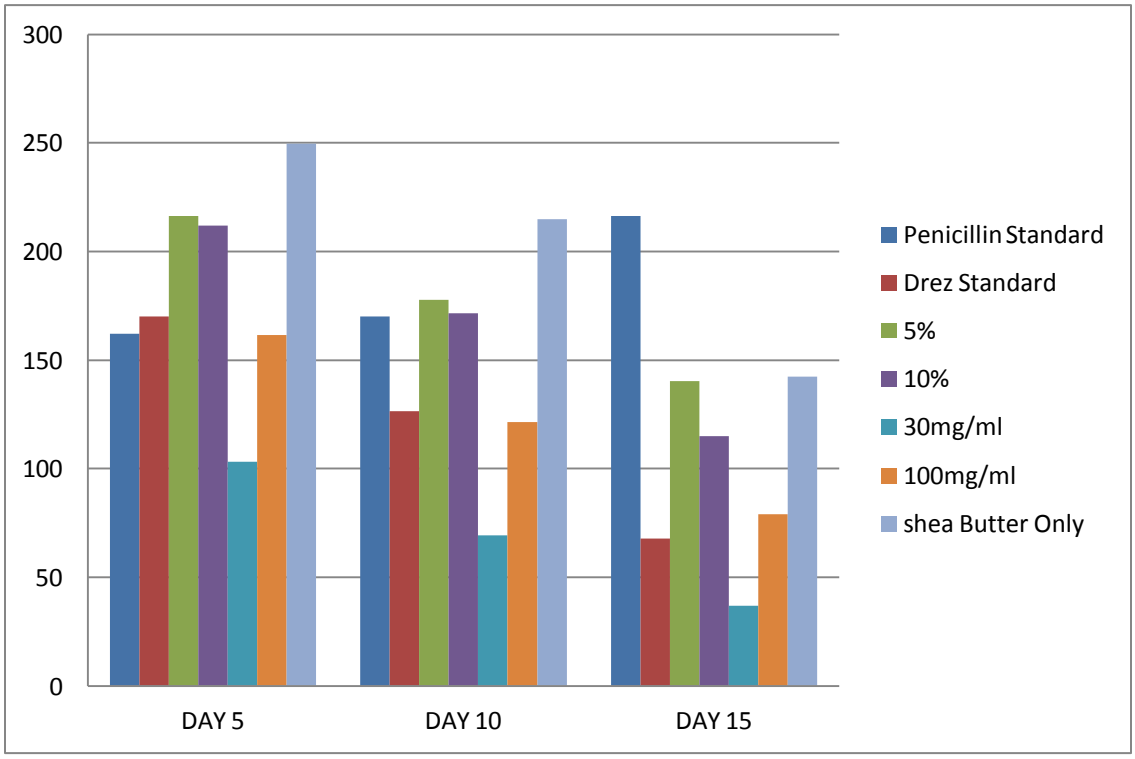

Figure 1. Comparison of the microbial load of the various rat groups that were giving different treatment. (Each bar indicates microbial load of different treated wound on the given days).

Tannins are known to promote wound healing due to their antimicrobial and astringent property which is responsible for wound contraction (Getie *et al*., 2002). The presence of tannin confirms haemostasis which is a property for wound healing activity in *A. spinosus*. Flavonoids, apart from contributing to the brilliant multi-colour for most plants (Sofowora, 1993), are noted to possess antioxidant and anti-inflammatory properties (Akuodor et al., 2010) which aid in the closure and acceleration of wound healing. Terpenoids are known to promote the wound healing process, mainly due to their astringent and antimicrobial properties, which seem to be responsible for wound contraction (Scortichini, 1991). Terpenoids may have great antifungal or antimicrobial potential due to possible effect on the non-

mevalonate pathway. This pathway is essential in fungi, protozoans, gram-negative bacteria and other micro-organisms for the synthesis of cell membrane components, prenylation proteins and as a secondary source of carbon (Nayak et. al., 2010). Studies with other plant materials also demonstrated the presence of similar phytochemical constituents, which were responsible for promoting wound healing activity in rats (Nayak and Cecropia, 2006).

Conclusion

To conclude, the various root preparations of *Amaranthus spinosus* exhibited a good wound healing effect comparable to those of the standards (Penicillin and Iodinated-Povidone with Metronidazole), standard antibiotics used in wound healing. The fixed oil also indicated an appreciable level of antimicrobial activity. These findings thus, justify the use of *A. spinosus* in folkloric medicine for wound healing.

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