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Evaluation of wound healing activity of *Achidium ohioense* (Schimp. ex Mull) oil on albino rats

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ABSTRACT

The study evaluated *Archidium ohioense* oil potential in wound management with three different wound models in rats. The rats were subjected to incision, excision and dead space models measuring 6 cm, 500 mm^2 and 1 cm, respectively. *A. ohioense* oil (0.5 and 1.0 mg/kg bwt) was administered topically, once daily for 10 days (incision and dead space models) or for 21 days or more (excision wound model) in rats. *In vivo* studies on wound breaking strength, rate of contraction, period of epithelization (excision model), granulation tissue free radicals (nitric oxide and lipid peroxidation), antioxidants [catalase (CAT) superoxide dismutase (SOD) and reduced glutathione (GSH)], connective tissue markers (hydroxyproline, hexosamine and hexuronic acid) and deep tissue histology (dead space model) were investigated. The results obtained reveal that *A. ohioense* oil enhanced wound breaking strength and contraction rate, tissue collagen formation and early epithelization period. There was decrease in the levels of free radical, enhanced antioxidant and connective tissue markers alongside histological substantiation of formation of more collagen in skin and deeper connective tissue. The findings of this study indicate that *A. ohioense* oil possessed wound healing activity when applied topically.

INTRODUCTION

Injuries that end in a break or an interruption within the anatomical structure and performance of living tissues which can be caused by physical, chemical, microbiological or immunological assaults are termed wound (Avinash and Priyanka, 2013; Dwajani and Gurumurthy, 2013). Wounds is an expressive burden both on patients and health care professionals worldwide because not only do they affect the physical and mental health of millions of people, it is also a challenging clinical problem with severe complications involving high cost of therapy which presents a frequent cause of morbidity and mortality (Velnar et al., 2009; Badri and Renu, 2011).

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Wound healing is the physical restoration of organ structure and performance characterized by cellular and biochemical processes. These processes occur in an organized and ordered pattern lasting from days to months or maybe years (Harari, 2004; 2003). There phases involved in wound healing include hemostasis, inflammatory, proliferative, and remodeling and maturation phase (Shalu et al., 2016).

Archidium ohioense is a tropical moss plant that belongs to the family of *Archidiaceae* and grows largely on rocky terrains. It is distributed geographically from coastal antarctica to the tundra of the northern hemisphere and from the Australian deserts to the Amazon rainforests. It is a common plant in South-West region of Nigeria. However, it is not used in traditional medicine because it seems insignificant (Babatunde, 2002).

MATERIALS AND METHODS

Plant Material collection and identification

A. ohioense used in this study was collected from Hill II at the Obafemi Awolowo University, Campus, Ile-Ife, (07°.30`N, 04°40`E) Amos and Aina (2009) in the months of July to September. The plant was identified and authenticated by a taxonomist at the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria; and a voucher specimen (IFE-17406) was deposited at IFE Herbarium. The *A. ohioense* plant was collected, sun dried and powdered by mechanical grinder.

Experimental animals

One hundred and five (105) healthy Albino rats of either sex were obtained from Faculty of Basic Medical Science, Obafemi Awolowo University, Ile-Ife, Nigeria. The animals were housed in standard plastic cages under laboratory conditions at the Animal House of the Department of Anatomy, Faculty of Basic Medical Sciences, O. A. U, Ile-Ife. The rats were allowed to acclimatize for two weeks and were fed with standard feeds (Ladokun Feeds, Ibadan) and watered *ad libitum*. All animal experiments complied with the 'Principles of Laboratory Animal Care' (NIH publication No. 82-23 revised in 2011).

Reagents and chemicals

All the reagents used were of analytical grades. The reagent/chemicals were obtained from various sources such as British Drug House (BDH) Chemicals Limited, London, Sigma Chemicals Limited, St. Louis, Mo., U.S.A. All solutions and buffers were prepared with glass distilled water and stored in the refrigerator.

Preparation of the methanol extract of A. ohioense

The method of Akinpelu et al. (2008) was adopted for the preparation of the plant extracts. Five kilograms (5 kg) of the powdered plant was soaked in 80% (v/v) methanol for three days with occasional stirring. The suspension was filtered with a double layered muslin cloth to obtain the methanolic extract which was allowed to stand overnight. The clear liquid portion was separated *in vacuo* at 35°C on a rotary evaporator (Edwards Vacuum Components, Crawley England) and lyophilized to allow for crude methanol extract.

Fractionation of crude methanol extract of A. ohioense

The method of Adeoye and Oyedapo (2004) was used in the partitioning. The methanol (180 g) was dissolved in 150 mL of hot distilled water in a 1000 mL separating funnel partitioned with n-hexane (200 mL × 3). The n-hexane fraction was concentrated *in vacuo* at 35°C to obtain a viscous and oily substance with amber colour. The oil was dried over anhydrous sodium sulphate (Na_2SO_4).

Acute dermal toxicity test

Acute dermal toxicity test was carried out as described by Mulisa et al. (2015). Ten healthy (male and female) Wistar rats of average weight 198±2.8 g were housed individually in a cage and acclimatized to the laboratory condition for 2 weeks prior to the test. Following the acclimatization, 10% of the animals' fur was shaved from the dorsal trunk of the test animals to the caudal region. A test limit dose of 2000 mg/kg bwt of the *A. ohioense* of oil was applied uniformly over the shaved area for 24 h, thereafter the residue of the test substance was removed and the animals were monitored daily for any sign of wound infection or skin abnormalities for 14 days.

Wound healing evaluation of incision wound model

Incision wound was created on Albino rats by procedure that was based on earlier methods of Agarwal et al. (2009) and Murthy et al. (2013). Typically, the dorsal fur of the animals was shaved. Two parallel 6 cm paravertebral incisions were made through the skin, one cm lateral to the midline of vertebral column after administering anesthesia. The wounds were closed by interrupted sutured 1 cm apart. *A. ohioense* oil was applied topically to groups 2 (0.5 mg/kg bwt) and 3 (1.0 mg/kg bwt) rats while gentamicin to groups 4 (0.5 mg/kg bwt) and 5 (1.0 mg/kg bwt) wounds once daily. The sutures were removed on 7^{th} post wounding day under a light anesthesia. Application of the test samples to the wounds continued till 9^{th} day. The wound breaking strength was estimated on the 10^{th} post wounding day.

Determination of wound breaking strength

On the 10^{th} day, the wound breaking strength was measured after anaesthetization by the constant water flow method (Lee, 1968). A line was drawn at 3 mm away from the wound on either side of the incision line. Two Allis forceps that faced each other were firmly applied on to the line with one forcep fixed, while the other was connected to a freely suspended light weight poly-propylene graduated container through a string run over to a pulley. Water was allowed to flow continuously and steadily into the container which transmitted gradual increase in weight to the wound site pulling apart the

wound edges. The water flow was stopped as soon as the wound opened up, and the volume of water collected in the container was recorded. The procedure was repeated on the wound on the contra lateral side.

Excision wound model

Excision wound was created on the Wistar rats according to the method of Morton and Malon (1972) as described by Shivananda et al. (2007). The animals were divided into four groups of five rats each and anaesthetized prior to creation of wounds with ketamine hydrochloride (10 mg/kg bwt) intravenously. The dorsal fur of the animals was shaved and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless-steel stencil. A full thickness of the excision wound of 2.5 cm in width (4.90 cm^2) and 0.2 cm depth was cut off along the markings. The entire wound was left open. The animals were then divided into four groups of five rats each.

Group 1: Control group rats were left untreated;
Group 2: Rats were treated with 0.5 mg/kg bwt *A. ohioense* oil extract;
Group 3: Rats were treated with 1.0 mg/kg bwt *A. ohioense* oil extract;
Group 4: Rats were treated with gentamicin ointment (0.5 mg/kg bwt); and
Group 5: Rats were treated with gentamicin ointment (1.0 mg/kg bwt).

The animals were treated topically on daily basis for 10 days.

Measurement of wound area

The wound size was measured daily during the change of dressing using sterile transparent cellophane paper and a permanent marker. For the wound size measurement, the perimeter of the wound was traced out on the cellophane paper with marker and the area of the traced outline was determined using a graph paper. The period of epithelialization was then calculated as the number of days required for falling off the dead tissue remnants without any residual raw wound (Shivananda et al., 2007).

Wound contraction was calculated as a percentage of the original wound size. Then progressive decrease in wound size was monitored. The percentage wound contraction for all the animals on the tenth day was calculated as described by Snowden (1981):

$$\frac{\text{Healed area on } 10^{th} \text{ day}}{\text{Total wound area}} \times 100\ mm^2$$

Healed area = original wound area - wound area on 10^{th} day

The period of epithelization was measured in days required for the eschar tissue to fall off leaving no raw wound behind.

Dead space wound model

Dead space wound model was carried out as described by Ilango and Chitra (2010). The rats were anesthetized with ketamine hydrochloride (10 mg/kg bwt) intra-venously, and then the fur in the dorsolumbar part was shaved. Incision (1 cm) was made on the shaved dorsolumbar part of the back and two polypropylene (0.5 × 2.5 cm^3) was then inserted in the dead space of the lumbar region on each side of the rats and was sutured uninterruptedly with a silk suture and then grouped as follows:

Group 1: Control group were left untreated;
Group 2: Rats were treated with 0.5 mg/kg bwt *A. ohioense* oil extract;
Group 3: Rats were treated with 1.0 mg/kg bwt *A. ohioense* oil extract;
Group 4: Rats were treated with gentamicin ointment (0.5 mg/kg bwt); and
Group 5: Rats were treated with gentamicin ointment (1.0 mg/kg bwt).

The oil and gentamicin ointment were applied topically on the wound site on the 10^{th} day, the rats were euthanized through cervical dislocation, an incision was made on the skin to the implanted tube. Then implanted tubes were carefully removed; granulation tissues were collected and weighed. The 10% (w/v) granulation tissue homogenates were prepared using 10 mM phosphate buffered saline (PBS) pH 7.4 at 4°C. The homogenate was centrifuge at 4000 rpm for 10 min. The supernatant was carefully decanted and kept in the refrigerator for further analysis. The remaining granulation tissue was weighed and dried at 50°C for 24 h and the final weight was recorded.

Biochemical assays

Estimation of lipid peroxidation level

The total amount of lipid peroxidation products in the granulation tissue samples was estimated by the thiobarbituric acid (TBA) reaction method which measures the malondialdehyde (MDA) reactive products according to the method of Ohkawa et al. (1979). Briefly, 0.5 mL tissue homogenate was mixed with 0.5 mL of 0.1 M phosphate buffer, pH 8.0 and 0.5 mL of 24% (w/v)

trichloroacetic acid (TCA), mixed thoroughly, incubated at room temperature for 10 min and followed by centrifugation at 3000 rpm for 20 min.

To 1 mL of supernatant was added 0.25 mL of 0.33% thiobarbituric acid in 20% (v/v) acetic acid and the resulting mixture was boil at 95°C for 1 h and cooled. The absorbance of the product was read at 532 nm against the reagent blank. The concentration of MDA was estimated using the expression:

$$\mathrm{MDA}(\mu\mathrm{M}) = \frac{A\ Sample}{\mathrm{l}\ \mathrm{X}\ \varepsilon}$$

l = Light path = 1 cm
ε = Molar absorptivity = 1.56 X 105 M-1 cm-1

Estimation of nitric oxide concentration

The assay was designed to measure the concentration of NO production following reduction of nitrate to nitrite using improved Griess reaction method (Grisham et al., 1996). Briefly to 500 µL granulation tissue homogenate was added 50 µL TCA in 10% (w/v) in 1.5 mL tubes. The resulting suspension was centrifuged for 10 min at 4000 rpm. The clear supernatant was transferred to clean tubes. Typically, 10 µL of each supernatant in separate, labeled tubes was added 0.1 mL of 1% sulphanilamide in 5% phosphoric acid and incubated at room temperature for 10 min, followed by addition of 0.5 mL 0.1% N-1-napthylethylenediaminedihydrochloride and incubated for 10 min at 60°C. The absorbance of the chromophore was measured at 546 nm against reagent blank. The standard calibration curve was prepared using sodium nitrite (0–10 µM). The amount of NO in the sample was extrapolated from the standard curve.

Estimation of antioxidant activities in the granulation tissues

Assay of catalase activity: The assay procedure of Aebi (1984) was used for catalase activity which was based on the ability of the enzyme to oxidize hydrogen peroxide. Fifty microliters (50 µL) of the granulation tissue homogenate was added 450 µL of 0.1 M phosphate buffer (pH 7.4) in a cuvette and followed by the addition of 500 µL of 20 mM H_2O_2. The change in absorbance at 240 nm was monitored at 30 s interval over a period of 3 min. The molar extinction coefficient of H_2O_2 (ε=43.6 M cm^{-1}) was used to determine the catalase activity. One unit of catalase activity was defined as 1 mmol of H_2O_2 degraded per minute and was expressed as units per milligram of protein using the formula below:

$$\mathrm{Units/ml} = \frac{\Delta \mathrm{A/min\ x\ df\ x\ TV}}{\mathrm{SV\ x\ 0.0436}}$$

Where: df, Dilution of original sample for catalase reaction; SV, sample volume in catalase reaction (mL); 0.0436, ε^{mM} for hydrogen peroxide; TV, total reaction volume.

Assay of superoxide dismutase (SOD) activity: The method of Mccord and Fridovich (1969) was applied for the assay of SOD activity in the granulation tissue was based on the inhibition of the formation of NADH-phenazinemethosulphate-nitroblue tetrazolium formazan. Typically, tissue homogenate (200 µL), 2.5 mL of 75 mM of Tris–HCl buffer pH 8.2, 30 mM EDTA and 300 µL of 2 mM of pyrogallol were mixed. The increase in absorbance was monitored at 420 nm for 3 min at 30 s interval. One unit of enzyme activity is 50% inhibition of the rate of autoxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD was expressed as unit/mg protein using the formula below:

Increase in absorbance per minutes = $A_3 – A_0/2.5$

Where A_0, absorbance after 30 s; A_3 = absorbance after 150 s.

% inhibition = 100 – (100 × S/B)

S, Increase in absorbance for substrate; B, increase in absorbance for blank.

Estimation of reduced glutathione (GSH) activity: The level of reduced glutathione was quantified according to the method of Beutler and Kelly (1963) as modified by Moron et al. (1979). Typically, 0.1 mL of granulation homogenate was mixed with 2 mL of 10% TCA heated at 50°C for 10 min and followed by centrifugation at 3 rpm for 5 min, then 1.0 mL of supernatant was mixed with 2.0 mL of 0.2 M phosphate buffer, pH 8.0 and 0.5 mL of 10 mM of Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) orDTNB] and mixed thoroughly. The absorbance was read at 412 nm against the reagent blank. The level of GSH was extrapolated from the standard calibration curve of GSH (0 – 100 µg/mL); and expressed as GSH µg/100 g tissue.

Estimation of concentration of granulation tissue total protein: The protein concentration was determined quantitatively described by Shacterk and Pollack (1973). Briefly, into clean and dry test-tubes in triplicates was pipetted 1.0 mL tissue homogenate, 1.0 mL of copper reagent (10% Na_2CO_3, 0.1% Na-Ktartarate and 0.05% $CuSO_4.5H_2O$) was added and mixed. The mixtures were left to stand for 10 min and then 4.0 mL of Folins – Ciocalteuphenol, solution was added. The blank was also prepared similarly with distilled water. The mixture was then incubated at 55°C for 5 min, removed and cooled

rapidly with water. The absorbance was read at 650 nm against the blank. The protein concentrations were calculated as:

$$\text{Conc. of protein (g/dl)} = \frac{\text{Abs. of sample}}{\text{Abs. of Std.}} \times \text{conc. of Std. protein}$$

Abs, Absorbance; conc., concentration; Std, standard.

Assessment of connective tissue parameters: Typically, granulation tissues (250 mg) was dried for 24 h at 50°C, weighed and transferred into glass stoppered test tube. Dry tissue (40 mg) was digested with 6 N HCl (1 mL) in a boiling water bath for 12 h. The hydrolysate was cooled and excess acid was neutralized with 10 N NaOH. The neutralized hydrolysate was diluted with distilled water to a concentration of 10 mg/mL. The concentrations of hydroxyproline, hexosamine, and hexuronic acid were estimated from final hydrolysate.

Estimation of hydroxyproline concentration

The method of Neuman and Logan (1950) was adopted for hydroxyproline assay. Neutralized hydrolysate (0.3 mL) served as the test sample for the assay. Pipetted in succession into each test tube were 0.3 mL each of hydrolysate, 0.3 mL of copper sulphate, 2.5 N sodium hydroxide and 6% hydrogen peroxide. The solution in each test tube was mixed and shaken occasionally after which the test tubes were placed in water bath at 80°C for 15 min with frequent shaking. The test tubes were chilled in ice and water. Into each of the test tubes, 1.2 mL of 3 N H_2SO_4 was added with agitation. p-Dimethylaminobenzaldehyde solution (0.6 mL) was then added to each of the test tubes and the content was thoroughly mixed. Into clean and dry test-tubes in triplicates 0.3 mL of the neutralized hydrolysate was also added, 0.3 mL of hydroxylproline standard (50 – 300 µL), the blank was also prepared using ns 0.3 mL of distilled water. Hydroxyproline was then read at 540 nm wavelength. The amount of hydroxyproline measured in microgram in 0.3 mL of test sample was extrapolated on the standard curve.

Estimation of hexosamine concentration

The method of Dische and Borenfreund (1950) was adopted for hexosamine estimation. Each neutralized hydrolysate (0.05 mL) served as the test sample for the assay. Test tubes were racked in triplicates, the reacting mixture was made up of 0.05 mL of hydrolyzed fraction diluted to 0.5 ml with distilled water and 0.5 mL of acetyl-acetone reagent. The mixture was heated in boiling water bath for 20 min then cooled under tap water. To this was added 1.5 mL of 95% alcohol, followed by an addition of 0.5 mL of Ehrlich's reagent. The reaction was allowed for 30 min to complete. Into clean and dry test-tubes in triplicates 0.3 mL of the neutralized hydrolysate was also added, 0.3 mL hexosamine standard (50 – 500 µL), the blank was also prepared using 0.3 mL of distilled water. Color intensity was measured at 530 nm against the blank. Hexosamine contained in the samples was extrapolated from the standard curve prepared with D (+) glucosamine hydrochloride.

Estimation of hexuronic acid concentration

The method of Bitter and Muir (1962) was adopted for hexuronic acid estimation. Exactly 2.5 mL of 0.025 M Borax in concentrated sulphuric acid was placed in stoppered tubes fixed in a rack and cooled to 4°C. Hydrolysate (0.125 mL) was made up to 0.5 mL with distilled water. Thereafter, the hydrolysate (0.5 mL) was layered on Borax-sulphuric acid mixture kept in rack at 4°C. The tubes were closed with glass stoppers and vortexed slowly initially, then vigorously, with constant cooling by placing tubes in ice container. The reacting mixture was heated for 10 min in a vigorously boiling water bath and cooled to room temperature. Thereafter, 0.1 mL of 0.125% carbazole reagent in absolute alcohol was added to each tube, vortexed and heated in the boiling water bath for further 15 min. The mixture was then cooled at room temperature and color intensity was measured at 530 nm against the blank. Hexuronic acid content of the samples was extrapolated from the standard curve prepared with D (+) glucurono-6, 3 lactone, from 5 to 40 µg/0.5 mL using 100 µg/mL working solution.

Histopathotological analysis of wound tissue

The tissue samples were fixed in Bouin's solution dehydrated through increasing grades of ethanol and then embedded in paraffin wax. The tissues were then cut to 5 µm sections with a rotary microtome, deparaffinized, mounted on clean glass slides and stained with hematoxylin and eosin (HE). The glass slides were then observed under the microscope for histomorphological changes.

Statistical analysis

Values were expressed as mean ± SEM for five rats in each group. Significant differences between the control and treated groups were determined by One-way

Analysis of Variance (ANOVA), using Graph Padprism 5. $p<0.05$ was considered to be significant.

RESULTS

Yield of methanol extract and oil of *A. ohioense*

A. ohioense whole plant (5 kg) yielded 70.52 g methanol extract which represented 1.4% (w/v) of the starting material. An odourless, amber coloured- oily substance (30 g) was obtained from the methanolic extract representing 0.6% of the starting material.

Determination of acute dermal toxicity

Maximum concentration of the *A. ohioense* oil administered at a limit dose of 2000 mg/kg was found to be safe. After 24 h of application of the oil, the site did not show any sign of inflammation. No overt symptoms of toxicity were observed when the animals were monitored for 48 h. Also, no changes in general behavioural pattern and mortality were recorded during the 14 days cage side observation.

Wound healing patterns in incision wound models

The results of the incision wound model revealed that *A. ohionse* oil (0.5 and 1.0 mg/kg bwt) and standard drug - gentamicin (0.5 and 1.0 mg/kg bwt) treated animals showed significant ($p<0.05$) increase in wound breaking strength when compared to the control (Figure 1). The wound breaking strength for *A. ohioense* oil (0.5 and 1 mg/kg bwt) and gentamicin ointment (0.5 and 1 mg/kg bwt) were 1515 ± 143.93g, 647.67 ± 62.10g and 1275 ± 89.27, 677.08 ± 72.56 g respectively, whereas the wound breaking strength for control group was 589.17±37.51g. Thus, the increase in wound breaking strength in extract treated groups' showed accelerated healing effect of *A. ohioense* oil in incision wounds.

Rate of wound contraction in excision wound models

The rate of wound contraction was measured as percentage reduction in original wound size at 5 days' interval using a millimeter scale graph paper. A better healing pattern was observed in rats treated with *A. ohioense*oil extract and gentamicin compared with the control group as shown in Figures 2 (A-E). There was a significant ($p<0.05$) reduction in wound length and the closure rate of rats treated with *A. ohioense* oil (0.5 mg/kg bwt) (B_1-B_3) when compared to *A. ohioense* (1.0 mg/kg bwt) (C_1-C_3), gentamicin (0.5 and 1.0 mg/kg bwt) (D_1–D_3 and E_1–E_3) and the control (A_1–A_3) on day 6. On day 1 to day 11 there were no significant reduction in the wound area (Table1) differences among the groups. On the 16th day of the study a significant reduction in the wound area was observed in *A. ohioense* oil [0.5 mg/kg bwt: (27.0 ± 7.28); 1.0 mg/kg bwt: (23.8 ± 5.77) and gentamicin (0.5 mg/kg bwt: (16.0 ± 4.0); 1.0 mg/kg bwt: (29.2 ± 2.88)] compared to control.

The average number of days that took for the shedding of eschar without leaving any residual raw wound (epithelialization period) was also significantly reduced in the *A. ohioense* oil ($p<0.05$) and standard drug treated groups ($p<0.05$) which was 22.0±1.00 for 0.5 mg/kg bwt, 22±0.52 for 1.0 mg/kg bwt, 21±0.60 for 0.5 mg/kg bwt and 21.4±0.17 for 1.0 mg/kg bwt respectively, when compared with control group (22.4±0.35).

The tensile strength exhibited by the *A. ohioense* oil (0.5 and 1.0 mg/kg bwt) and gentamicin ointment (0.5 and 1.0 mg/kg bwt) (was statistically significant ($p<0.05$) when compared to the control group.

Dead space wound model

Granulation tissue weights

In dead space wound model, the weights of dry and wet granulation tissue were significantly higher in *A. ohioense* oil (0.5 1.0 mg/kg bwt) treated group when compared with the control (Table 2). The wet granulation tissue of 1.0 mg/kg *A. ohioense* oil treated rat increased by 68.85% 1.0 while that of 0.5 mg/kg bwt *A. ohioense* oil, gentamicin 0.5 mg/kg bwt and 1.0 mg/kg bwt treated rats are 36. 80%, 29.65% and 26.90% respectively compared to control rats. Also, 50.92% of the weight of dry granulation tissue was recorded for 0.5 mg g/kg bwt of *A.ohioense* treated rats while there were significant increase of 46.49%, 35.46% and 41.18% in dry granulation tissue of 1.0 mg/kg bwt *A. ohioense* oil, gentamicin 0.5 mg/kg bwt and 1.0 mg/kg bwt treated rats of respectively when compared to the control.

Total protein levels

Granulation tissue total protein content in 0.5 and 1.0 mg/kg bwt *A. ohioense* oil treated rats were 1.18±0.23 (g/dl) and 0.88±0.09 (g/dl) respectively while that of 0.5 and 1.0 mg/kg bwt gentamicin treated rats were 1.13 ± 0.32 and 0.64±0.00(g/dl) respectively. The granulation tissue total protein content of both *A. ohioense* oil and gentamicin treated rats were significantly ($p<0.05$) higher compared to control rats 0.44±0.08 (g/dl).

Levels of antioxidant potentials

Estimation of granulation tissue CAT, SOD and GSH

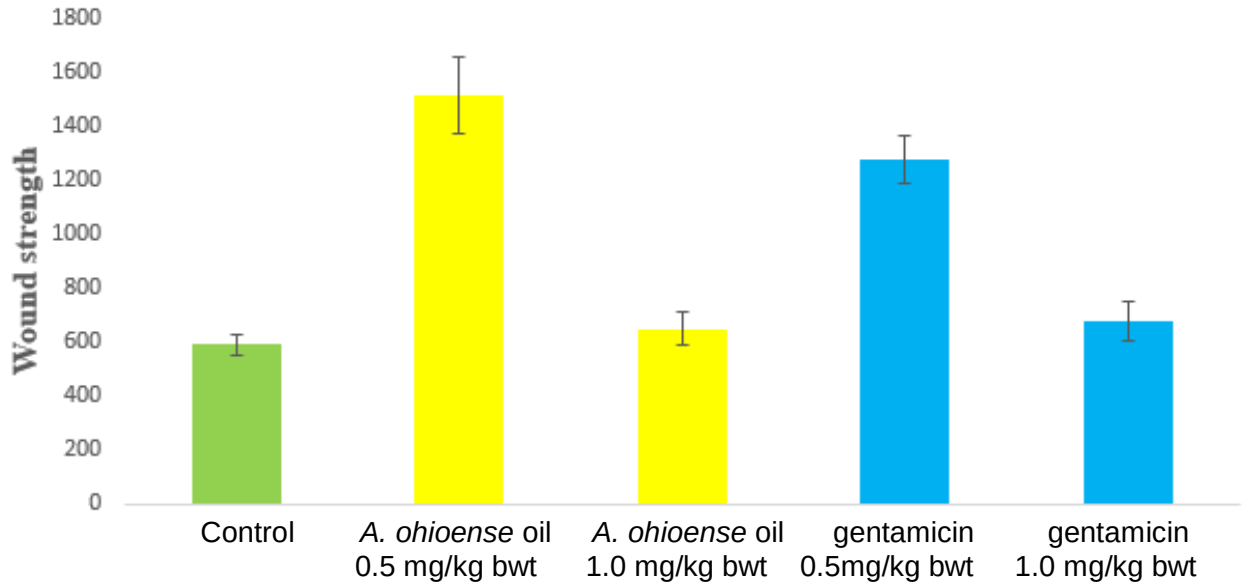

Figure 1. Wound breaking strength A. *ohioense* oil and gentamicin.

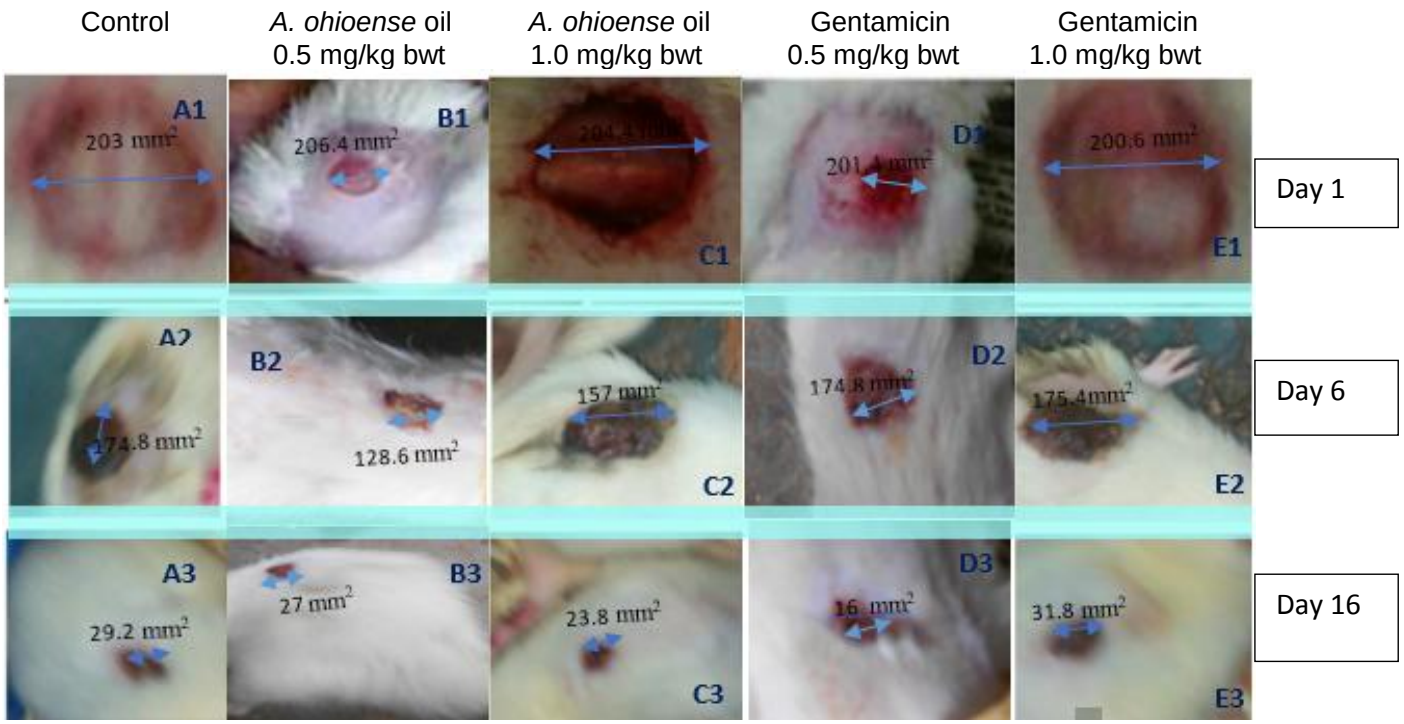

Figure 2. Shows wound area (mm) of different groups over a period of 16 days.
Control (A1 – A3); *A. ohioense*oil treated groups (B1- B3; C1-C3); gentamicin treated groups (D1- D3; E1- E3).

Table 1. Effect of *A. ohioense* oil and gentamicin on wound contraction in excision wound model.

Treatment	Wound area in mm^2/rat (% wound contraction)			
	Day 1	Day 6	Day 11	Day 16
Control	200.6±12.33 (0.00±0.00)	174.4±11.78 (14.85±4.43)	89.8±10.87 (47.05±11.85)	31.8±9.95 (81.25±19.37)
A. ohioense oil (0.5 mg/kg bwt)	206.4±7.92 (0.00±0.00)	128.6±7.14 (37.69±9.92)	53.2±6.50 (74.22±17.94)	27.0±7.28 (86.92±8.14)
A. ohioense oil (1 mg/kg bwt)	204.4±7.95 (0.00±0.00)	157±7.69 (24.89±3.19)	75.6±7.22 (60.29±9.16)	23.8±5.77 (87.5±27.38)
Gentamicin (0.5 mg/kg bwt)	193.4±11.91 (0.00±0.00)	166.8±10.89 (13.75±8.58)	60.2±10.93 (68.87±8.18)	16.0±4.01 (91.73±66.33)
Gentamicin (1 mg/kg bwt)	201.4±6.54 (0.00±0.00)	174.8±6.49 (14.27±0.75)	99.6±5.20 (50.98±20.52)	29.2±2.88 (85.63±56.01)

Values expressed as mean ± SEM from 5 animals in each group. Values in parenthesis represent % increase compared with control.

Table 2. Mean weight of wet and dry granulation tissue.

Groups	Wet tissue	Dry tissue
	(mg)	
Control	93.33±22.28	53.33±7.20
A. ohioense oi 0.5 mg/kg bwt	202±145*	164±4.56*
A. ohioense oi 1.0 mg/kg bwt	500.00±64.24*	146±12.65*
Gentamicin ointment 0.5 mg/kg bwt)	172±17.30*	112±9.55*
Gentamicin ointment 1.0 mg/kg bwt)	162.00±24*	128±21.08*

Values expressed as mean ± SEM from 5 rats in each group. *Significantly different from control ($p<0.05$).

Table 3. Effect of *A. ohioense* oil on antioxidant enzymes.

Groups	CAT	SOD
	(nM/mg protein)	
Control	0.55±0.002	0.58±0.06
A. ohioense oil 0.5 mg/kg bwt	0.071±0.005*	0.86±0.08
A. ohioense oil 1.0 mg/kg bwt	0.70±0.009*	0.87±0.04
Gentamicin ointment 0.5 mg/kg bwt	0.62±0.003	0.73±0.06
Gentamicin ointment 1.0 mg/kg bwt	0.72±0.006*	0.95±0.03

Values expressed as mean ± SEM from 5 rats in each group. *Significantly different from control ($p<0.05$).

Table 4. Effect of *A. ohioense* oil on NO, MDA and GSH.

Groups	MDA	NO	GSH
		(nM/mg protein)	
Control	1.23±1.10	0.83±0.54	0.09±0.005
A. ohioense oil 0.5 mg/kg bwt	4.97±2.31	0.33±0.19**	0.13±0.02
A. ohioense oil 1.0 mg/kg bwt	1.22±1.09	0.67±0.17*	0.11±0.005
Gentamicin ointment 0.5 mg/kg bwt	4.96±3.67	0.53±0.31*	0.10±0.01
Gentamicin ointment 1.0 mg/kg bwt	1.21±0.96.	0.76±0.32*	0.23±0.008*

Values expressed as mean ± SEM from 5 rats in each group significantly different from control ($p<0.05$).

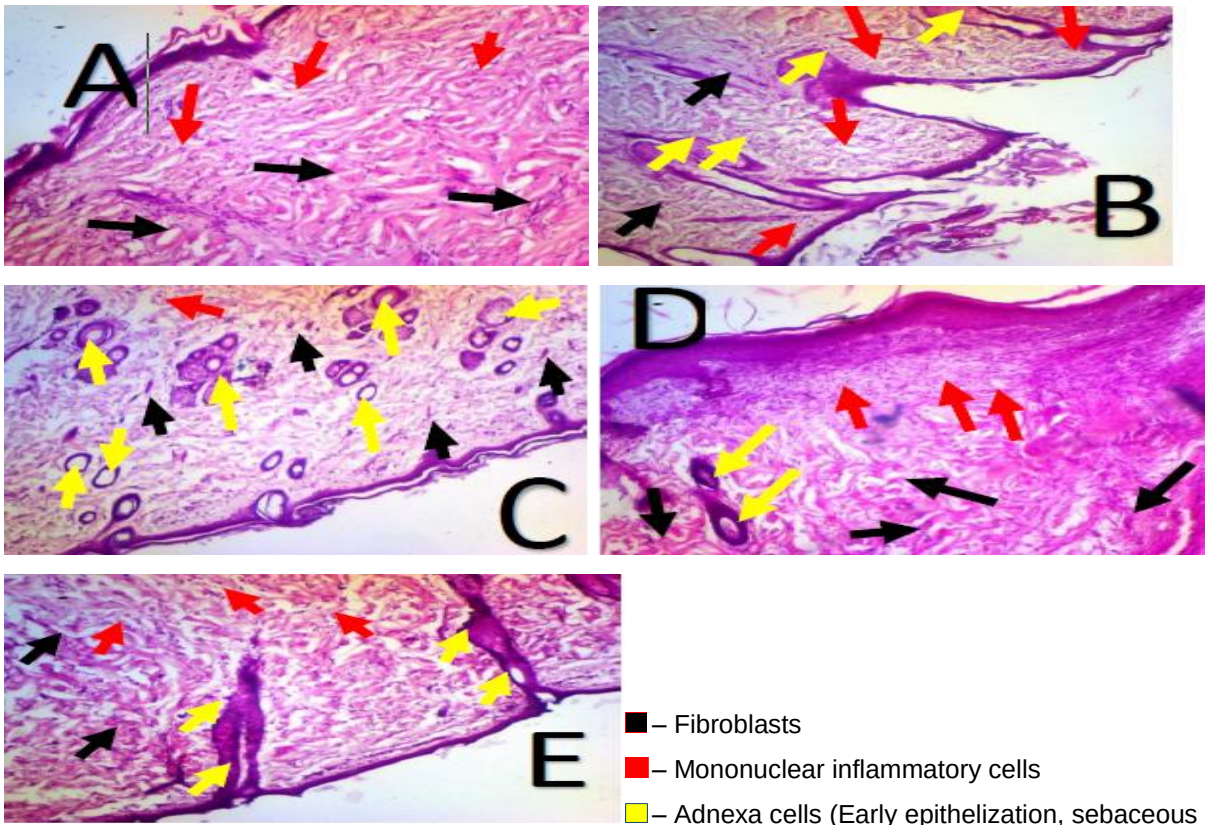

Figure 3. (A –E): Histopathology of granulation tissue at day 16 with H & E X400. **A,** Granulation tissue of control rat showed mononuclear inflammatory cells and fibroblasts; **B,** granulation tissue of 0.5 mg/kg bwt *A. ohioense* oil treated rat adnexa cells, suggesting early epithelization; **C,** granulation tissue of 1.0 mg/kg bwt *A. ohioense* oil treated showing high level of neovascularization and vast evidence of early epithelization; **D,** granulation tissue of 0.5 mg/kg bwt gentamicin treated showing adnexa cells, an indication of early epithelization; **E,** granulation tissue of 1.0 mg/kg bwt gentamicin treated showing neovascularization and an indication of early epithelization.

levels showed increase in the levels of these antioxidant biomolecules in *A. ohioense* and gentamicin treated rats compared to control group (Table 3). Significant increase in the level of CAT was observed in rats treated with *A. ohioense* 0.5 mg/kg bwt and 1.0 mg/kg bwt while GSH level significantly increased in 1.0 mg/kg bwt gentamicin treated rats.

Free radicals

The measurement of the effect of *A. ohioense* oil and standard drug (gentamicin ointment) on malondialdehyde (MDA) and nitric oxide (NO) in wet granulation tissue was as shown in Table 4. The MDA level in the control group was significantly higher at $p<0.05$ compared to the *A. ohioense* oil treated group. Treatment with *A. ohioense* oil significantly reduced nitric oxide level when compared to the control and compared favourably with gentamicin ointment.

Connective tissue markers

Table 5 is the summary of connective tissue markers content of wet granulation tissue. Hydroxylproline level was observed to increase significantly ($p<0.05$) in *A. ohioense* treated rats when compared with the control. Hexuronic acid and hexosamine levels were significantly higher in 0.5 mg/kg bwt compared to 1.0 mg/kg bwt *A. ohioense* oil treated rats compared to control group. The results on connective tissue markers with *A. ohioense* oil were comparable with gentamicin ointment.

Histological studies

Histomorphological analysis of excision wound model healed skin

Figure 4 shows the photomicrographs of granulation tissue of deeper structure of control rats (Figure 3A) with

Table 5. Effect of *A. ohioense* oil on connective tissue markers.

Groups	Hydroxylprolin	Hexuronic acid	Hexosamine
		(µg/mg protein)	
Control	20.59±1.83	10.08±0.84	194.75±24.89
A. ohioense oil 0.5 mg/kg bwt	38.18±0.17	16.09±0.26*	433.56±2.86*
A. ohioense oil 1.0 mg/kg bwt	48.21±0.65*	15.97±0.64*	220.74±31.47
Gentamicin ointment 0.5 mg/kg bwt	38.63±0.25*	16.30±0.21*	464.30±1.17*
Gentamicin ointment 1.0 mg/kg bwt	30.37±0.70*	19.67±0.81*	341.85±48.58

Values expressed as mean ± SEM from 5 animals in each group. *Significantly different from control ($p < 0.05$).

mononuclear inflammatory cells, scattered fibroblasts (minimal fibrosis), and few proliferating vasculature in granulation tissue, while the granulation tissue of rats treated with *A. ohioense* oil (0.5 and 1.0 mg/kg bwt; Figures 3B and C) and gentamicin (0.5 and 1.0 mg/kg bwt; Figures 3D and E) showed abundance of eosinophilic collagen tissue and neovascularisation with few inflammatory cells indicative of healing fibrosis.

Estimation collagen fibres

Figure 4 shows result of differential staining for collagen and elastic fibres deposition across the different groups using image J analyses. The percentage colour intensity of collagen and elastic fibres deposition at the wound sites across the groups increased in the treated rats in following order control < *A. ohioense* oil 0.5 mg/kg bwt < gentamicin 0.5 mg/kg bwt < gentamicin 1.0 mg/kg bwt < *A. ohioense* oil 1.0 mg/kg bwt < *A. ohioense* oil 1.0 mg/kg.

Estimation reticulinfibres

The percentage colour intensity of reticulinfibres (type III collagen) deposition at the wound sites across the groups using image J analyses was as shown in Figure 5. All treated groups showed higher levels of reticulinfibres deposition compared with the control with *A. ohieonse* oil 1.0 mg/kg bwt treated rats exhibiting highest reticulinfibres deposition.

DISCUSSION

Wound constitutes a major health problem, both in terms of illness and death. Wound healing is a complex multistep process that involves epithelization, contraction, and connective tissue deposition. Delayed or non-healing of the wound has brought attention to promote wound healing process (Gio and Dipietro, 2010).

The result obtained from acute dermal toxicity study showed that topical application *A. ohioense* oil extract did not produce any abnormalities. In incision wound model, increased in wound breaking strength observed in *A. ohioense* oil treated rats compared to untreated rats could be attributed to increase in collagen deposition and stabilization. The wound breaking strength is acquired from both collagen synthesis and the maturation process. At the initial stage, wound possessed little strength thereafter, the strength of the wound increased rapidly as collagen deposition increases and cross-linkages are formed between the collagen fibers (Ramachandra, 2012). Collagen is one of the key protein of the extracellular matrix involved in scar formation during the healing of connective tissues (Udupa et al., 2006). The increased in wound tensile strength in the treated rats was further supported by histological results which showed higher concentration of collagen fibres [including reticulinfibres (type III collagen)] deposition at the wound site.

In excision wound model, topical application of *A. ohioense* oil and gentamicin significantly enhanced wound closure and reduced epithelization period when compared with control group rats. Wound contraction is the movement caused by the proliferation and transformation of fibroblasts into myfibroblasts facilitates re-epithelization by shortening the migrating distance of keratinocytes as improvement of wound healing activity had been connected with reduction in the period of epithelization (Süntar et al., 2010; Gupta, 2008; Strodtbeck, 2001).

The significant reduction of epithelization period observed in the *A. ohioense* oil treated rats might be attributed to enhancement of cellular proliferation by the oil extract. Likewise, the anti-inflammatory effect of the oil bioactive compounds such as hexadecane and 9, 12-octadecadienoic acid methyl ester perhaps substantiated the reduction in inflammatory cells as shown in histopathology of granulation tissue of deeper structure of the treated cells (Anyim et al., 2015).

Hydroxyproline is a non-essential amino acid derivative found in collagen. Monitoring the content of

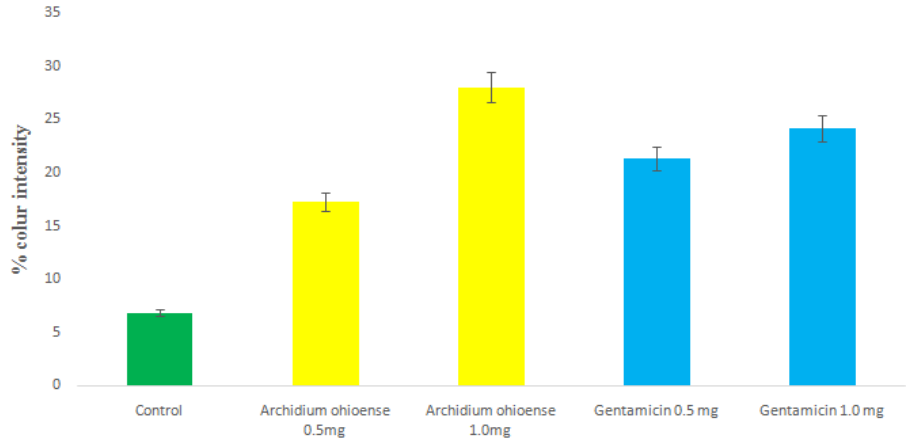

Figure 4. Image J analyses of percentage colour intensity of collagen fibres deposition at the wound sites across the groups.

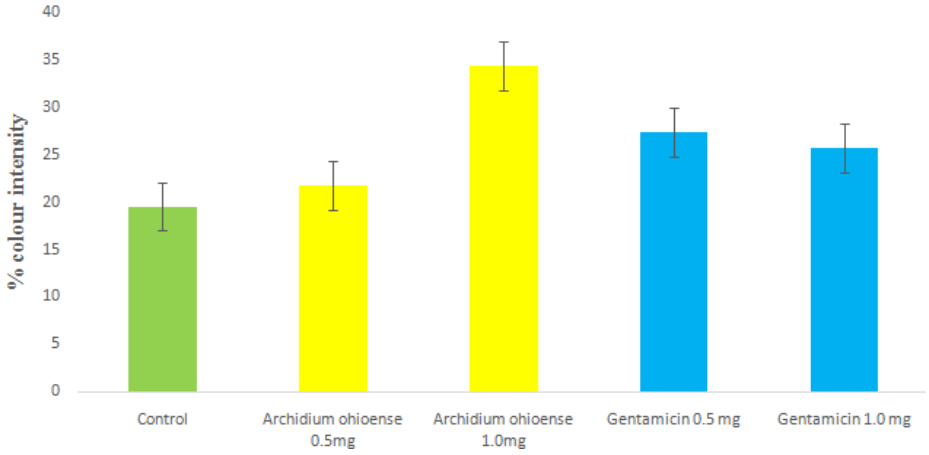

Figure 5. Image J analyses of percentage colour intensity of reticulinfibres (type III collagen) deposition at the wound sites across the groups.

hydroxyproline in healing tissue estimates its collagen turnover (Nayak et al., 2006). The increased inhydroxyproline content of granulation tissue in dead space wound model of *A. ohioense* oil treated rats indicated faster collagen turnover which probably leads to rapid healing with concurrent increase in tensile strength (Roy et al., 2006). Hexosamine and hexuronic acids act as ground substrata in the matrix molecules in order to stabilize the collagen fibres by improving electrostatic and ionic interactions within it and possibly control their eventual alignment and distinctive size (Trowbridge and Gallo, 2002). Thus, significant increase in hexuronic acid and hexosamine levels observed in *A. ohioense* oil treated rats' granulation tissue indicate collagen fibers stabilization and contribute to boosted tensile strength.

Antioxidants prevent tissue damaged induced by free radicals by scavenging, enhance decomposition or preventing the synthesis of the free radicals (Lobo et al., 2010). Therefore, estimation of antioxidants like GSH, SOD and CAT in wound models is pertinent. In the study, *A. ohioense* oil treated rats showed appreciable increase in the activities of SOD, CAT and GSH thus suggesting *A. ohioense* oil as a promising agent in oxidative damage prevention and wound healing process acceleration. Moreover, significant reduction in nitric oxide level was observed in *A. ohioense* oil treated animals while lipid peroxidative product (MDA) was slightly reduced (though not significant) only in 1.0 mg/kg bwt in *A. ohioense* oil and gentamicin treated animals compared to control. It could be surmised that scavenging of free radicals could be one of the mechanisms by which *A. ohioense* oil elicits its wound healing activity.

In dead space wound model, increase in wet and dry granulation tissue weights in *A. ohioense* oil treated rats

suggested that *A. ohioense* oil is capable of healing dead space wounds. Increased in protein per gram of tissue was also noted in the oil treated rats which is evident by the increase in antioxidant enzymes and connective tissue markers. A deficiency of protein had been implicated in impairment of capillary formation, fibroblast proliferation, proteoglycan synthesis, collagen synthesis; wound remodeling and decreased leukocyte phagocytosis (Gogia, 1995).

Histomorpology of the healed skin sections of *A. ohioense* oil and standard reference drug treated rats in dead space wound excised on 10th day post wounding showed proliferation of adnexa and absence of acanthosis. Reduction of mononuclear inflammatory cells, increased in fibrocollagenous cells, a near-normal epidermis and dermis were also observed. All these established advanced wound healing and early epithelization in treated rats compared to the control. It was noted that in gentamicin treated rats, epidermis and dermis were not as near normal as observed in *A. ohioense* oil (1.0 mg/kg bwt). Fibroblasts, one of the most abundant cell types in connective tissues, are responsible for maintenance of skin homeostasis and orchestrating physiological tissue repair. In injured tissue, fibroblasts become activated, acquired alpha smooth muscle actin expression and differentiate into myofibroblasts that synthesized extra cellular matrix components (Darby et al., 2014). Both fibroblasts and myofibroblasts generate traction and contractile forces, respectively, which are beneficial to tissue repair (Li and Wang, 2011; Hinz, 2007). Histopathological results further substantiated that *A. ohioense* oil also facilitate increase in the rate of wound contraction by promoting production of fibroblasts around the wound area thereby pulling the skin closed (www.advancedtissue.com/2015/12/wound-care-tip-for-proliferation-phase-wound-healing, 2015).

In conclusion, the present study showed that *A. ohioense* oil exhibited wound healing activities evidenced by enhanced tensile strength, wound contraction rate, antioxidant enzymes production, collagen fibres deposition, connective tissue constituent formation and decreased free radical generated around the wound area.

However, further study to isolate and characterized the bioactive compound(s) in the oil that might be responsible for biological activity of *A. ohioense* oil wound healing.

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