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Evaluation of Wound Healing Activity of Crude Latex Obtained From Calotropis Procera Stem

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ABSTRACT
Evaluation of wound healing activity of crude latex of Calotropis procera stem was investigated in this present study. Thirty (30) rats of both sexes were divided into six groups of five animals each, Group A was administered distilled water only; group B was treated with 0% Calotropis procera crude latex + 100% ointment ; group C was treated with 25% C. Calotropis procera crude latex + 75% ointment; group D was treated with 50% Calotropis procera crude latex + 50% ointment; group E was treated with 75% Calotropis procera crude latex + 25% ointment and group F was treated with 100% Calotropis procera crude latex + 0% ointment. In In-vitro studies; the data was expressed as average cell number, the more the concentration of crude latex of Calotropis procera plant the more the average cell number. Excision wound model showed significant increase ($P<0.05$) in percentage wound closure in groups treated with latex (C to F). On day 16 increased wound closure was observed with increase in amount of stem latex mostly group F with mean value of 100.00±2.27%. Least wound closure on mean value of 45.63±4.48% was observed in group administered distilled water only. in-vitro studies (average number of cell), incision wound model (tensile strength) and hydroxyproline also showed significant increase ($p>0.05$) with increased concentration of crude latex. Hydroxyproline enhances the production of collagen, an essential component of the extracellular matrix required for wound healing. From the present study, the crude latex of Calotropis procera stem was observed to possess important phytochemicals with angiogenesis and fibrogenesis properties. Further research works on identification and isolation of bioactive component(s) that are responsible for the wound healing activity in the crude latex of Calotropis procera stem are recommended.

Keywords: Calotropis procera, latex, wound healing, phytochemicals, hydroxylproline.

I. INTRODUCTION

Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissues. Wound may be accidental or as a result of planned or deliberate surgical incision (Narendra et al., 2009). Wound healing is a complex series of cellular events that brings about the restoration of the anatomic structure and function of damaged part of the body. Healing of wound is a biological process that is initiated by trauma and often terminated by scar formation (Narendra et al., 2009). The process of wound healing occurs in different phases such as coagulation, epithelization, granulation, collegenation and tissue remodeling. At the time of wounding, the initial inflammatory phase occurs with the activation of the coagulation cascade that causes the release of cytokines which in turn stimulate chemotaxis of neutrophils and macrophages into the wound to begin early debridement (Wen-Hsiang et al., 2010). In wound healing mechanism following the migration of platelets, the first response cells, neutrophils and macrophages migrate to the wound. Numerous enzymes and cytokines are secreted by macrophages and neutrophils. Among this tumor necrosis factor (TNF) is the one which stimulates the angiogenesis, helps to build up the tissue granulation bed and thus has significant potential to improve the healing process (Al-Qarawi et al., 2001). Plants may exert their effect by modulating the cytokine(s) secretion during different conditions. TNF- is a major cytokine secreted by macrophages and neutrophils during the inflammation phase (Al-Qarawi et al., 2001). Several medicinal agents of natural origin have been used for the treatment of wounds and keloids.

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Examples include Ocimum sanctum, Ocimum gratissimum, alba, Eucalyptus globulus, Calotropis procera (Prasanna et al., 2007). Calotropis procera (family: Asclepiadaceae), referred to as Calotropis, is native to South-West and South-East Asia and Africa and also occurs on the Caribbean Islands, in Central and South America. In complementary and alternative medicine, the whole plant of Calotropis, leaves, barks as well as its latex have been employed in the treatment and management of many health conditions such as jaundice, joint pains, fever, asthma, snake bite, malaria, dysmenorrhoea, eczema and leprosy (Parotta, 2001). The latex is soaked in cotton for application into dental cavity to treat toothache (Suresh et al., 2005), gum bleeding and for dressing fresh skin burns (Kumar and Arya, 2007).

Despite technological advancement, wound exudates management is still a clinical challenge. Thick or viscous exudates worsen the problem where most of the novel dressings do not possess the capabilities to manage the viscosity with exudate absorption (Tickle, 2012). Maceration to the peri-wound area is additional challenge, irrespective of availability of topical barrier application products and the innovative dressings to lock exudates (Tickle, 2012). Above this, non-availability of sufficient clinical report of the assessment and documentation of wound exudates worsen the problems for the selection and cost-effective dressings. Control and removal of scar formed after wound healing is also an emerging area of research (Visscher et al., 2014). The scar is formed due to collagen formation and it is an outcome of natural healing. The nature of scar depends on the inflammatory phase of the healing. The prolonged inflammatory phase results into excessive scar formation; whereas too less inflammation is not supporting the wound healing (Visscher et al., 2014).

Therefore, the aim of the study was to evaluate the wound healing activity of latex of Calotropis procera stem.

II. MATERIAL AND METHODS

MATERIALS

Collection of Experimental Animals

40 healthy Wistar albino rats (4 weeks old) of either sex with mean weight of 90.0±3.1g were obtained from the animal house of University of Nigeria Nsukka, Enugu State (Sharma and Sikarwar, 2008). The rats were divided into 6 cages of 5 rats same sex per cage for the wounding experiment and kept in the animal house of Biochemistry & Molecular Biology Department, Nasarawa State University, Keffi - Nigeria; the animals were fed ad libitum, allowed access to free food (Vital feed - growers mash) and water. They were acclimatized for 2 weeks in the new environment.

Collection of Plant sample

Modified procedure of Narendra et al., (2009) was used for collection of plant sample. The collection of fresh crude latex was done by making small incisions on stem of matured Calotropis procera plant plant within Nasarawa State University, Keffi, Nigeria, allowing the crude latex to flow into sterile plastic bottles. The crude latex was gently handled to maintain its integrity during transport to the laboratory. The crude latex was then refrigerated at 25^{o}C until needed.

METHODS

Determination of Quantitative and Qualitative Phytochemical Content

The procedures decribed by Okerulu et al., (2017) and Sofowora (1989) were used for the qualitative and quantitative determination of phytochemical contents of the latex of Calotropis procera stem.

Test for Tannins

About 0.1 g of crude latex sample was boiled in 4ml of water in a test tube and then filtered. Few drops of 0.1% ferric chloride were added to observe brownish green or blue-black coloration indicative of the presence of tannins.

Saponins

Emulsion test with the olive oil:

1ml of crude latex of Calotropis procera was poured in test tubes; shook dynamically to form a stable froth, followed by addition of six drops of olive oil to this sample. Formation of an emulsion revealed the presence of saponins.

Test for Flavonoids

About 3ml of dilute ammonia was added to 2ml of crude latex of Calotropis procera. This was followed by addition of 1ml concentrated Sulphuric acid (H_2SO_4). Yellow coloration in each extract showed the presence of flavonoids.

Steroids

10ml chloroform was added in 1ml of each extract in a test tube. After that 10ml concentrated sulphuric acid was dissolved in this test tube. Two layers were formed; lower layer expressed yellow color along green fluorescence while upper layer showed red. The formation of these layers indicates steroids were present.

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Test for Alkaloids

The residue obtained from the evaporation of 50ml of the crude latex of C. procera was titrated with 20ml of dilute hydrochloric acid and 0.5g of sodium chloride and filtered. The filtrate was rendered alkaline with ammonium hydroxide and then extracted with successive portions of chloroform. The combined chloroform extract evaporated to dryness, the residues dissolved in 2ml hydrochloric acid and tested with silico-tungestic acid and Mayer's reagents. The formed precipitate was, in each case, indicates the presence of primary, secondary and tertiary alkaloids. The aqueous alkaline layer was acidified with hydrochloric acid and tested with silico-tungestic acid and Mayer's reagents. A precipitate was formed indicates the presence of quaternary alkaloids.

Alkaloids

To 2ml of each fraction, 2ml of conc. HCl was added and then few drops of Mayer's reagent were mixed to it. Formation of white precipitate or green color indicated the presence of alkaloids.

Phenols

To the 1ml of crude latex of Calotropis procera, 2ml of distilled water and three drops 10 % $FeCl_3$ were added. Formation of blue green color was showing phenol presence.

Grouping of Experimental Animals

According to (Jones et al., 2004) animals were divided into 6 cage groups of 5 rats each for the wounding experiment.

Group A: This group was administered distilled water only

Group B: This group was treated with 0% C. procera crude latex + 100% Penicillin (0:4) mL

Group C: This group was treated with 25% C. procera latex + 75% Penicillin (1:3) mL

Group D: This group was treated with 50% C. procera latex + 50% Penicillin (1:1) mL

Group E: This group was treated with 75% C. procera latex + 25% Penicillin (3:1) mL

Group F: This group was treated with 100% C.procera latex + 0% Penicillin (4:0) mL

Incision Wounds Model

Incision wound determination was carried out according to procedures described by Narendra et al., (2009). The animals were anesthetized with slight vapour inhalation of diethyl ether and the skin of the rats was shaved and disinfected with 70% alcohol. Paravertebral long incision of 4 cm length was made through full thickness of the skin at a distance about 1.5 cm from the middle on right side of the back. The wounds were closed with interrupted sutures of 0.5 cm apart using sterile surgical thread (No. 000) and a curved needle (No. 11). On day 9, sutures were removed and the tensile strength of healed wounds was measured with a tensiometer (DY300, India) and calculated using the following formula:

Tensile strength = breaking strength/cross sectional area of skin

Determination of Excision Wounds Model

According to Narendra et al., (2009), the animals were anesthetized with slight vapor inhalation of diethyl ether and the right side of each rat was shaved and disinfected with 70% alcohol. Excision wounds sized 300 mm² and 2 mm depth was made by cutting out layer of skin from the shaven area using sterile surgical blade. The entire wound was left opened. The treatment was done topically in all the cases. The treatment lasted for 16 days of various concentrations. Wound areas was measured on days 1, 5, 9, 13 and 16 for all groups, using a transparency paper and a permanent marker over the wound and tracing it out. The parameter studied was percentage wound contraction calculated using the following formula: percent wound contraction = (original wound area - unhealed area)/original wound area ×100

Determination of wound healing test in-vitro

Transwell assay the migration activity of fibroblasts was performed using 24-well transwell chamber with 8.0 um pore polycarbonate filter inserts (Costar, Cambridge, MA, USA). Cells (3×10 cells/well) suspended in serum-free RPMI 1640 containing 0.1% BSA was overlaid in the upper chamber of each transwell. In each lower chamber, 500 ul of serum-free RPMI 1640 containing 0.1% BSA in the presence or absence of compounds was added. Then the inserts was incubated at 37°C in a humidified atmosphere containing 5% carbon (ii) oxide (CO) for 24h. The cells that had not penetrated the filters were removed using cotton swabs (Rasik et al., 1999). The migrated cells attached to the bottom side was fixed in 100 percent methanol for 10 min and stained with Wright-Giemsa. The cells migrated to the lower surface of the filter in five microscopic fields of X400 magnification was counted in each filter. Triplicate samples were acquired and the data was expressed as the average cell number of 15 fields.

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Determination of wound healing test in-vivo

In-vivo evaluation of wound healing was done according to the procedure described by Arya and Kumar (2004), 100% ointment was used, with 5 mg of latex with 50 mg of Vaseline. The cells was attached to the bottom side was fixed in 100 percent methanol for 10 min and stained with Wright-Giemsa. The cells migrated to the lower surface of the filter in five microscopic fields of x400 magnification was counted in each filter.

Determination of the hydroxyproline content

According to Xueling et al. (2013), the healed wound tissues was excised and analyzed for hydroxylproline content after excision wound on day 16, which is a basic constituent of collagen. Tissues were dried in a hot air oven at 60°C to constant weight and were hydrolysed in 6N HCl at 130°C for 4 hours in sealed tubes. The hydrolysate was neutralised to pH 7.0 and was subjected to Chloramine-T oxidation for 20 minutes. The reaction was terminated by addition of 0.4M perchloric acid and colour was developed with the help of Ehrlich reagent at 60°C and measured at 560 nm using a Spectrophotometer (V – 730, Jasco UAE).

STATISTICAL ANALYSIS

Data obtained were presented as Mean ± SEM of mean was value of the determinations, one-way analysis of variance (ANOVA) was used to test for significance at 5% confidence level.

III. RESULTS AND DISCUSSION

Quantitative and qualitative photochemical composition of crude latex of Calotropis procera

The results of quantitative and qualitative phytochemical analysis of crude latex of Calotropis procera stem are presented in Figure 1. The results showed the presence and amounts of the phytochemicals detected as trace (saponins, steroids) and much (alkaloids, tannins, flavonoids, phenols). The order of concentration from much to least of the phytochemicals were flavonoids (117.0 mg/100g) > tannins (91.2 mg/100g) > phenols (70.7 mg/100g) > alkaloids (61.0 mg/100g) > steroids (0.80 mg/100g) > saponins (0.30 mg/100g).

Excision wounds model (Percentage wound closure/contraction)

The result of percentage wound closure measured four times on four days intervals is presented in Figure 2. The result of day 5 showed that the mean values of 45.63 ± 4.48, 84.98 ± 5.95, 89.53 ± 5.95, 89.58 ± 1.79, 91.61 ± 2.79 and 100.00 ± 2.27 were recorded for groups A, B, C, D, E and F respectively. The percentage wound closure increased with increase in amount of Calotropis procera stem latex. The group A animals treated with only distilled water showed the least mean value of wound closure.

In-vitro studies (average cell number)

Figure 3 showed the result of in-vitro studies (average cell number) of crude latex of Calotropis procera stem. The result showed that the mean values of 2.34 ± 1.14, 4.68 ± 0.90, 5.13 ± 1.30, 5.48 ± 1.82, 7.79 ± 2.40 and 10.38 ± 4.57 were recorded for groups A, B, C, D, E and F respectively with least and high mean values recorded for groups A and F respectively.

Hydroxylproline and Incision wounds model (Tensile strength)

The results of incision wounds model (tensile strength) and hydroxylproline are presented in Figure 4 & 5. The mean values for hydroxyl proline and incision wound (tensile strength) were 10.46 ± 0.36; 3.99 ± 0.33, 11.08 ± 0.48; 4.63 ± 0.47, 11.09 ± 0.50; 4.86 ± 0.44, 11.42 ± 0.37; 4.83 ± 0.84, 11.74 ± 0.33; 5.18 ± 0.33 and 12.04 ± 0.82; 6.04 ± 0.15 were for groups A, B, C, D, E and F respectively. Throughout the experiment both incision wound and hydroxyproline mean values were found to increase in all treated groups.

Flavonoids was found to be highest, reported from previous study shows that flavonoids possess anti-viral, anti-inflammatory, antioxidant activity, cytotoxic and also used in the treatment of hypertension, diabetes, rheumatic fever (Usoh et al., 2005; Tolulope, 2007). Tannins have been reported as antibacterial aggents, tannins decrease bacterial proliferation. Phenols have potential for beneficial effects on health by blocking key enzymes at microbial metabolism. Alkaloids also interfere with cell division; hence the presence of alkaloids in the plant makes it a possible remedy in the treatment of cancer (Adejumo & Ajiboso, 2003).

Saponins are used as anti-fungi agent and also as industrial adjuvants (Adejumo & Ajiboso, 2003). Steroids were found to be low in concentration; steroids are used in the stimulation of bone marrow and growth. It stimulates lean body mass and also play vital roles in the prevention of bone loss in elderly men (Yamamato and Gaynor, 2000).

The in-vitro data was expressed as average cell number, the more the concentration of crude latex of Calotropis procera stem the more the average cell number hence the more the healing as

shown in the present study. Khare (2007) has reported that increase in average cell number as an indication of maintenance of a cell type-specific cell density which is important for cell function and developmental process.

Excision wound model showed significant increase in percentage wound closure with the treated group mostly group F, there was increase as wound edges move toward each other. According to Tejero-Trujeque (2001), contraction occurs when the wound edges move towards each other in a centripetal fashion thus reducing the wound's dimensions. Contraction involves a dynamic process where cells organize their surrounding tissue matrix to reduce normal healing time by shrinking the amount of extracellular matrix (ECM) that needs to be produced (Jones et al., 2004). Wound contraction is beneficial as it can significantly reduce healing time because less granulation tissue needs to be produced to replace tissue loss (Calvin, 1988). Hydroxyproline is also an important marker for wound healing.

Skin has been described as a flexible, tough and strong sensory organ which can be pulled and stretched to a great extent without damaging it (Satyanarayana and Chakrapani, 2011). In the present study, tensile strength increases as the concentration of crude latex increases. According to Moulin et al., (2000), the strength and flexibility of the skin comes from two structures found in the dermis layers: collagen and elastin. Collagen is a fibrous protein, and it gives skin strength and holds its various structures together. In contrast, elastin is a crenulated protein and it gives skin its flexibility and elasticity.

IV. CONCLUSION

The crude latex of Calotropis procera stem possessed important phytochemicals with angiogenesis and fibrogenesis properties as observed in its increased mean values of wound closure, cell number, tensile strength and hydroxyl proline.

RECOMMENDATIONS

Further research works on identification and isolation of bioactive component(s) that are responsible for the wound healing activity are recommended.

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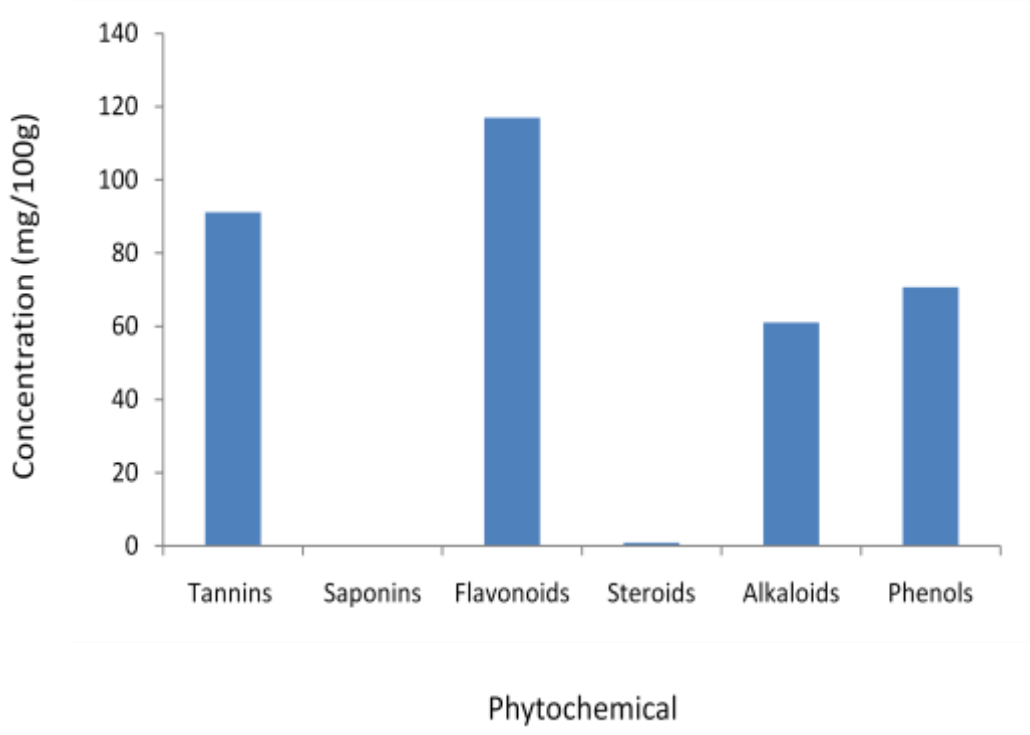

Figure 1: Mean values of concentration of phytochemicals in crude latex of Calotropis procera stem.

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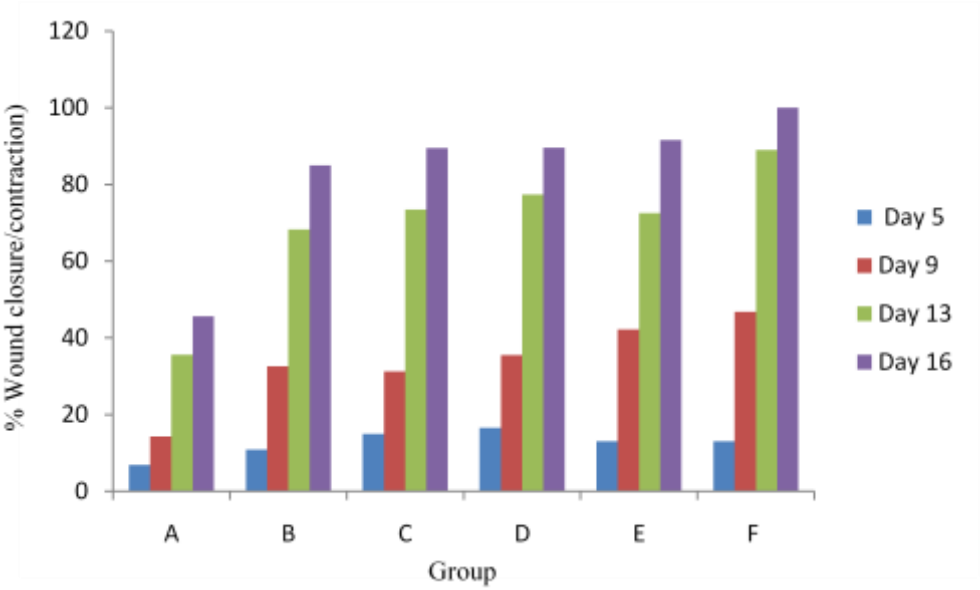

Figure 2: The mean values of percentage wound closure of treated and untreated groups.

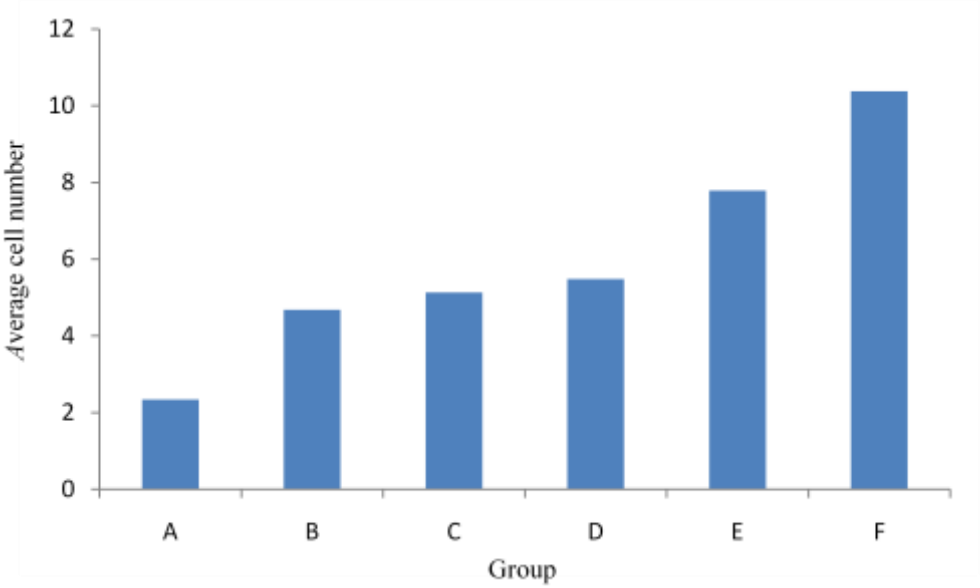

Figure 3: The mean values of in-vitro studies (average cell number) of crude latex of Calotropis procera stem.

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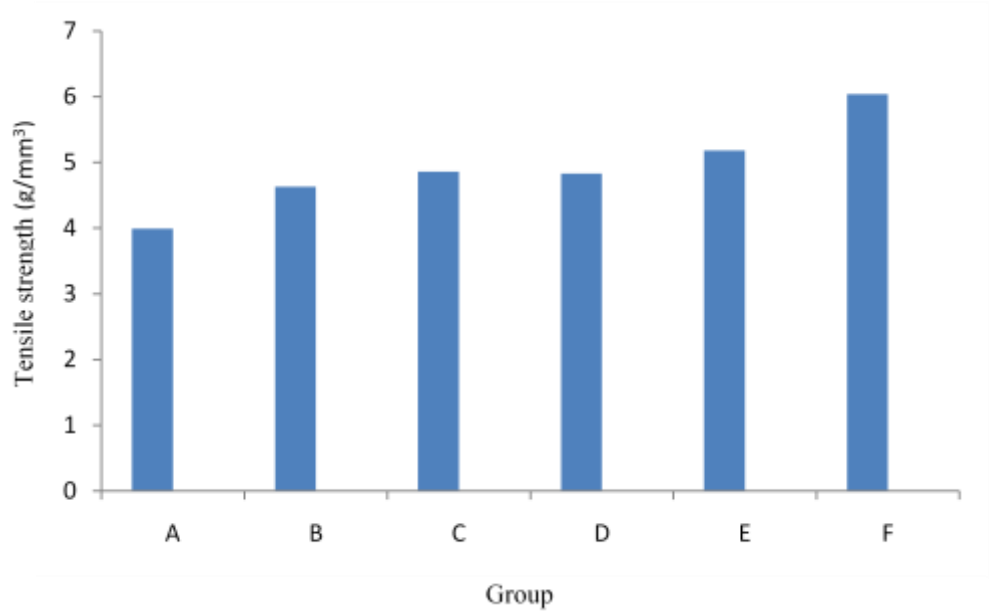

Figure 4: The mean values of incision wounds model (tensile strength) of treated and untreated groups.

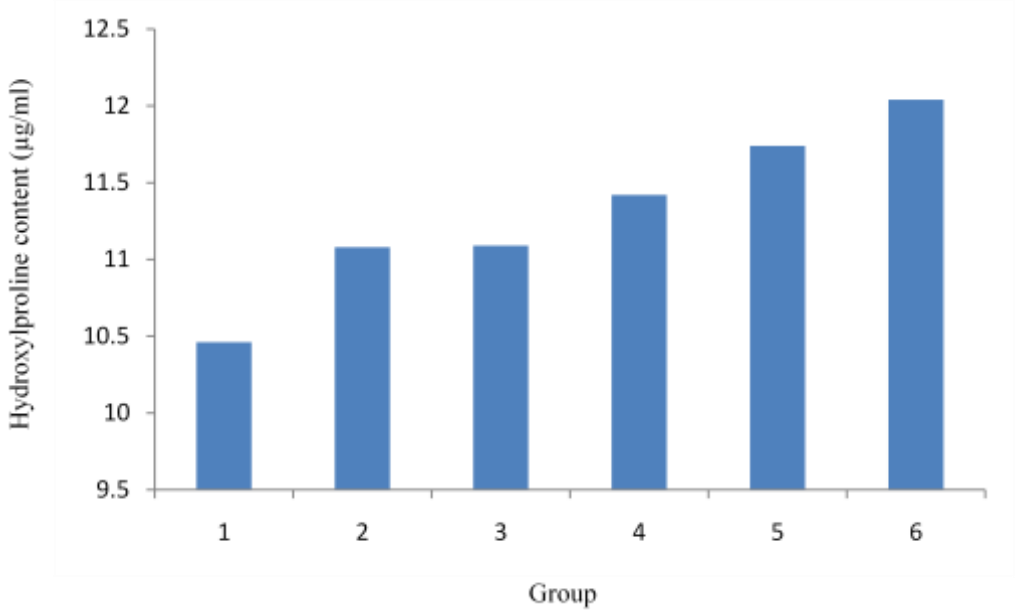

Figure 5: The mean values of hydroxylproline content of treated and untreated groups.