African walnut is used in Nigerian folkloric medicine for the treatment of bacterial infections and ailments caused by oxidative stress. The n-hexane and methanol extracts of cooked African walnut- Tetracarpidium conophorum (Mull Arg) was investigated to evaluate the rate of wound healing in Wistar rats. Wounds treated with placebo containing 5%, 10% T. conophorum extracts or gentamicin significantly (p = 0.05) accelerated the rate of wound healing compared to wounds treated with pure ointment. When compared to standard group, the percentage wound contraction on day 4 for 10% n-hexane group was significant while no significant (p = 0.05) different was observed in the wound contraction activity of the other groups below the 16th day. The percentage mean wound contraction on day 18 were 69.18, 84.14, 90.60, 88.36, 96.50 and 98.09, respectively, for the negative control, 5% n-hexane, 10% n-hexane 5% methanol, 10% methanol and gentamicin respectively. The present study thus establishes the dose-dependent wound healing activity of T. conophorum nuts, probably due to the inherent antioxidant and previously reported immunomodulatory activities.

**Keywords:** Tetracarpidium conophorum, Re-epithelization, Wound healing, Gentamicin
INTRODUCTION

Wounds are physical injuries that result in an opening or breaking of the skin and causes disturbance in the normal skin anatomy and function (Strodtbeck 2001). These result in the loss of continuity of epithelium with or without the loss of underlying connective tissue. Badly treated or untreated wound can become chronic and it represents huge burden in patient due to cost and duration of treatment. Wound is a major cause of morbidity affecting more than 1% of the United Kingdom (UK) population and with treatment cost of at least £1 million per year (Thomas and Hardings 2002). Wound healing is an intricate process in which the skin or other organ tissue repairs itself after injury. When the skin is injured, a set of complex biochemical events take place in a closely ordained cascade to repair the damage (Govindarajan et al. 2007). Within minutes of injury, platelet (thrombocytes) aggregates at the injury site to form a fibrin clot (which acts to control active bleeding and maintain hemostasis). A number of secondary metabolites or compounds isolated from plants have been demonstrated in animal models as active principles responsible for facilitating healing of wounds. Some of the most important ones include oleanolic acid, polysaccharides, gentiopicroside, sweroside, swertiamarin, shikonin derivatives (deoxyshikonin, acetyl shikonin, 3-hydroxy-isovaleryl shikonin, and 5,8-Odimethyl acetyl shikonin), asiaticoside, asiatic acid, madecassic, quercetin, isolectin, isorhamnetin, kaempferol, curcumin, sesamol (3,4 methylenedioxyphenol), coluteol, colutequinone B, hyperforin, catechins, and isoflavonoids (Karodi et al. 2009). *Tetraparpidium conophorum* (Family Euphorbiaceae) commonly called the African walnut is a perennial climbing shrub 10 - 20 feet long, found growing wild in forest zones of sub-Saharan Africa (Oluwole and Okusanya 1993). Studies have shown that the African walnut possess some properties that are required for wound healing like antibacterial (Ajaiyeba and Fadare 2006), antioxidant (Amaeze et al. 2011) and immunostimulating activities (Animashaun, Adetoro and Hughes 1994). Hence the present research is aimed at assessing the ability of these properties in accelerating wound healing on excision wound model in rats.

METHODS

Chemicals and Reagents
All the drugs and chemicals used in the study were of analytical grade. White soft paraffin (Placebo) was obtained from Boots Plc, UK while lignocaine HCl (2%, 100 mg/5 mL) was obtained from Mulberry Chemicals Pvt Ltd India. Methanol and n-hexane reagents were obtained from Sigma Chemicals (St. Louis, MO, USA).

Plant Material

The cooked African walnut (*Tetracarpidium conophorum*) was purchased from local market in Port Harcourt, Nigeria. The nuts were identified and authenticated by Mr. Osuala of the Department of Pharmacognosy, University of Port Harcourt, Nigeria. A voucher specimen (UPC 108505) of the sample was deposited in the herbarium of the department.

Preparation of Extract

Cooked nuts of *Tetracarpidium conophorum* were cut into pieces and air-dried. The dried nuts were ground into powder using Willey mill. Three hundred gram (300 g) of the pulverized powder was defatted with n-hexane (800 mL) for 48 h. The resultant oil content was collected using a separating funnel. The marc was dried and macerated with methanol (800 mL) for 48 h. The methanol extract was concentrated *in vacuo* at 40°C. The methanol extract was lyophilized by a freeze-dryer to produce powdered forms of the extract. To 100 g fused ointment base was added 5 g and 10 g methanol extract respectively on a clean white tile and the mixture was triturated carefully ensuring homogeneous mixing to get 5% and 10% w/w methanol extract formulation, also same method was used for 5 g and 10 g n-hexane extract respectively to get 5% and 10% w/w n-hexane extract formulation. A 1.0 g extract formulations was applied topically onto each of the animal wound and the wound healing progression observed.

Photochemical Screening of the Extracts

The phytochemical screening of the extracts included the test for alkaloids, glycosides, terpenoids, saponins, tannins, flavonoids, steroids and triterpenes (Harborne 1984).
**Acute Toxicity Test**

An acute toxic test was of the methanol extract was done to determine the safe dose for the extract according to previously described method (Lorke 1983). Nine mice of both sexes were subdivided equally into 3 groups and treated with 0.01, 0.10, and 1.0 g/kg body weight dose of the extract preparation. Mice were deprived of food for overnight but not water prior dosing. Food was not allowed for a further 3 to 4 h after dosing. Observations on behavioral changes and mortality of the animals were done following treatment for 24 h. In absence of death, 3 mice were treated with 1.6, 2.9 and 5 g/kg body weight of the extract. The acute toxicity LD₅₀ was calculated indicating that *T. conophorum* causes no lethality at all.

**Ointment Formulation and Topical Application**

The ointments were formulated with white soft paraffin as ointment base using the fusion method (Cooper and Gunn 1987). Six different products were formulated and applied topically to wound areas according to the groupings.

*Group 1: Served as vehicle control and applied pure ointment.*

*Group 2: 5%, w/w, n-hexane extract ointment is applied*

*Group 3: 10% w/w, n-hexane extract ointment is applied*

*Group 4: 5%, w/w, methanol extract ointment is applied*

*Group 5: 10%, w/w, methanol extract ointment is applied*

*Group 6: 2%, w/w gentamicin ointment applied (positive control).*

The animals were treated topically by applying 1.0 g of the extracts formulation on the wound twice daily. The wounds were observed daily until complete wound-healing enclosure occurs. The experiment was carried out under aseptic condition.

**In Vivo Wound Healing Evaluation**

**Experimental Animals**
Wistar Rats of both sexes were obtained from the Department of Pharmacology, University of Port Harcourt. The rats were divided randomly into 6 groups of 5 rats each. Rats with body weight between 180 - 200 g were selected for the experiment. The animals were maintained on standard pellet diet and tap water. The use and care of laboratory animals were conducted in accordance with internationally accepted best practices as contained in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and approved by the local Ethics Committee of our institution. The animals received treatments topically, with each group receiving appropriate formulation.

**Excision Wound Model**

Wound infliction on the back of the animals was done by removing hairs using a shaving machine. The wounds were induced under anaesthesia using 1 mL lignocaine HCl (2%, 100 mg/5 mL) subcutaneously around the area under investigation. The excision wound was created according to the reported method (Odoh and Ezugwu 2007) with slight modification. Excision wound was inflicted on the dorsal thoracic region (1.5 cm away from the vertebral column on either side and 5 cm away from ear) using a round seal of 2.5 cm diameter. The skin of impressed area was excised to the full thickness to obtain a wound area of between 200-350 mm² diameter and 2 mm depth. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. After haemostasis was achieved using normal saline, the animals were housed in different cages and allowed free access to feed and water.

**Wound Area Measurement**

Wound contraction was assessed by tracing the wound area on a transparent graph paper from which the wound surface area was evaluated on day 0, 4, 8, 12, 16 and 18. The evaluated wound surface was then employed to calculate the percentage of wound contraction taking the initial size of the wound as 100%. The period of re-epithelialization was also observed for each of the treatment group. Percentage wound contraction was determined using:
\[
\text{\% Wound contraction} = \frac{\text{Wound area on day 0} - \text{wound area on Nth day}}{\text{Wound area on day 0}} \times 100
\]

Nth day = 4, 8, 12, 16 and 18th post wounding days.

Statistical analysis

The data were expressed as mean ± SEM of at least triplicate determinations (n = 3). To demonstrate statistical significance of data, a One-way Analysis of Variance (ANOVA) using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA) was performed followed by Dunnett’s posthoc test. Differences between test and control treatments are considered significant at \( P = 0.05 \).

RESULTS

Several concentrations range from 5 -10% w/w of the \( n \)-hexane and methanol extracts of \textit{T. conophorum} were evaluated for their wound healing activity in rats using \textit{in vivo} models. It was observed that wound healing ability of the extracts were concentration dependent.

Percentage yields of the extracts

The percentage yield of the \( n \)-hexane extract was 6.67% while that of the methanol extract was 3.33%.

Phytochemical Analysis

The preliminary photochemical screening of \textit{Tetracarpidium conophorum} extracts showed presence of alkaloids, saponins, glycosides, flavonoids and tannins in the methanol extract while steroids, terpenoids and triterpenes were present in the \( n \)-hexane extract as shown in Table 1.
Acute Toxicity

The acute toxicity test of the methanol extract showed that the extract was safe with lethality value (LD₅₀) of above 5.0 g/kg body weight.

Wound Healing Ability

The wound healing activity of *T. conophorum* nuts extracts is shown in Figure 2. A faster healing pattern of wound closure was observed in rats treated with the extracts and gentamicin within 8 days compared to the control rats (Figure 3). There was a significant reduction in wound area from day the fourth day of treatment with the closure rate much faster than when compared with control rats (Figure 4). Significant wound contraction was observed in the test group from day 8 in 10% *n*-hexane extract group (p=0.05) with the rest groups showing significant contraction from day 12. Also, the 10% *n*-hexane and methanol groups were the only groups that showed significant wound contraction on day 18 (p=0.05).

Reepithelialization of Wounds

The reepithelialization period in all the treatment groups is shown in Figure 1. The standard group had the least period of epithelization (19 days) while the negative control group had the longest period (28 days). The reepithelialization of the test group rats, were higher than that of the positive group rats (21 – 25 days).

DISCUSSION

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 (Abdull *et al.* 2010). The aim in these processes is to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin. Healing process, a natural body reaction to injury, initiates immediately after wounding and occurs in four stages. The first phase is coagulation which controls excessive blood loss from the damaged vessels. The next stage of the healing process is inflammation and debridement of wound followed by reepithelialisation which includes
proliferation, migration and differentiation of squamous epithelial cells of the epidermis. In the final stage of the healing process, collagen deposition and remodeling occurs within the dermis (Phillips, Whitehe & Kinghton 1991). Wounds require treatment to either shorten the time for healing or to minimize the undesired consequences (Rajinder et al. 2008). Treatment requires that attention be directed towards discovering an agent, which will accelerate wound healing either when it is progressing normally or when it is suppressed by various agents like corticosteroids, anti-neoplastics or non-steroidal anti-inflammatory agents and in disease conditions.

The T. conophorum nut extracts exhibited significant wound healing activity as compared to the negative control in excision wound model. It is observed that the wound contracting ability of the extracts ointment treated groups showed significant \( p=0.05 \) wound healing from day 4 day onwards. The wound closure of the extracts ointment treated group decreases as the treatment days increases showing significant wound contraction as shown in Figure 3.

Wound contraction, a part of the proliferative phase of wound healing, occurs through the centripetal movement of the tissues surrounding the wound, which is mediated by myofibroblasts. The myofibroblasts establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells (Midwood, William and Schwarzbauer 2004). Wound contraction or retraction (wound shrinking process), depends on the tissue’s reparative abilities, type and damage extent and tissue general health state (Danielle et al. 2012). In both excision and burn wound the wound healing progression are monitored; and as such there could be comparison between the two types of wound in this present study (Pawar, Chaurasiya and Jain 2013). The increased wound contraction in the treated groups may be due to the enhanced activity of fibroblasts in T. conophorum nut extracts.

The period of reepithelialization expressed as the number of days required for falling of the eschar (dead- tissue remnants) without any residual raw wound (Bhat, Shankrappa and Shivakumar 2007). Epithelialization is necessary in the repair of all type of wounds (Bhat, Shankrappa and Shivakumar 2007).

A sharp decrease in the period of reepithelialization was observed in the positive control group (19 days) as against that observed in the negative control group (28 days). When compared with positive control, the reepithelialization time was lower in the treatment group.
This is consistent with a previous study (Danielle et al. 2012). The shorter period taking for the wound contraction and reepithelialization in the group treated with standard drug could be attributed to the antimicrobial activity (Sabath 2006) drug.

The wound healing activity observed in the extracts could be attributed to the presence of secondary metabolites in the nuts. The preliminary photochemical analysis showed that the methanol extract contains active constituents that are needed by the body for wound healing. Our findings showed that *T. conophorum* contains active ingredients that are needed by our body such as flavonoid and tannin. It could be conceivable that the *T. conophorum* extracts exert their wound healing activity through the flavonoids since flavonoids are reported to improve wound healing and protect tissues from oxidative damage (Saurez, Herreta and Marhuenda 2009).

Tannins present in the methanol extract of the nut may be responsible for its wound healing ability (Rashed, Afifi and Disi 1996). Tannins have been reported to possess wound healing action by improving regeneration and organization of the new tissue (Liete et al. 2002).

The presence of triterpenoids and saponins in the extracts appear to be responsible for wound contraction and elevated rate of epithelialization. Flavonoids also possess potent antioxidant and free radical-scavenging effect, enhancing the level of antioxidant enzymes in granuloma tissue (Shenoy et al. 2009).

The extraction of total alkaloids and tannins from *Tetracarpidium conophorum* (Nigerian walnut) seeds have been reported (Ayoola, Onawumi and Faboya 2011). Also, the phytochemical analysis of the aqueous extracts of the seeds of *T. conophorum* has been investigated (Uche, Obianime and Aprioku 2010). Their result revealed the presence of flavonoids, tannins, carbohydrate, alkaloids, terpenoids, steroids, volatile oils, saponins and cardiac glycosides in the seed. This was consistent with our findings considering that water and methanol has approximate polarities.

**CONCLUSION**

The results obtained in our present study clearly indicate that the extracts of *Tetracarpidium conophorum* had significant wound healing activity in rats. The wound healing effect of extracts of *T. conophorum* may be due to the presence of one or more active secondary metabolites. The
enhanced capacity of wound healing of the plant could be explained on the basis of antioxidant and immunostimulating activities of the plant that are well documented in the literature.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Osuala of the Department of Pharmacognosy, University of Port Harcourt, Nigeria for the identification of the nuts.

REFERENCES


<table>
<thead>
<tr>
<th>Constituents</th>
<th>Methanol extract</th>
<th>n-hexane extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: + = present, - = absence
Fig. 1: Reepithelialization period in all treatment groups
Fig. 3: Wound Contraction in experimental animals

Fig. 4: Percentage Wound Contraction in treated animals