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WOUND HEALING ACTIVITY OF N-HEXANE AND METHANOL EXTRACTS OF *TETRACARPIDIUM CONOPHORUM* (MULL. ARG.) HUTCH (AFRICAN WALNUT) IN WISTAR RATS

KENNETH MADUABUCHI EZEALISIJI[1], ABAYOMI EBENEZAR OMOTOSHO[1], RUTH UDOH[1] AND MATTHIAS ONYEBUCHI AGBO[2*]

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Port Harcourt, 32001 Rivers State, Nigeria
[2]Department of Pharmaceutical and Medicinal Chemistry, University of Nigeria, Nsukka, 410001 Enugu State, Nigeria

The 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.) were investigated to evaluate the rate of wound healing in Wistar rats. Treatment of wounds with placebo (5%, 10% T. conophorum extracts or gentamicin) significantly (p=0.05) showed accelerated rate of healing compared with that of the pure ointment. When compared to the standard group, the percentage of wound contraction on day 4 was significant for the 10% n-hexane group, while no significant (p=0.05) difference was observed in the wound contraction activity of the other groups before the 16th day. The percentages of 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 extracts of the T. conophorum nut, most likely due to their inherent antioxidant and immunomodulatory activities.

Keywords: *Tetracarpidium conophorum*, Re-epithelialisation, Wound healing, Gentamicin

INTRODUCTION

Physical injuries that results in an opening or breaking of skin called wounds are caused by disruption in the skin anatomy and function (Strodtbeck 2001). Wound is usually accompanied by loss of epithelia tissues with or without loss of the connective tissue. Badly treated or untreated wounds can become chronic and this represents a huge burden on the patient due to the cost and duration of treatment. Wounds are a major cause of morbidity, affecting more than 1% of the United Kingdom population with treatment costs of at least £ 1 million per year (Thomas and Harding 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 takes place in a closely ordained cascade to repair the damage (Govindarajan *et al*. 2007). The mechanism of wound healing results from aggregation of platelets (thrombocytes) at the site of injury to form a clot. A number of secondary metabolites like oleanolic acid, polysaccharides, shikonic derivatives and asiaticosides have been reported to possess wound healing properties (Karodi *et al*. 2009). *Tetracarpidium conophorum* (Family Euphorbiaceae), commonly called the African walnut, is a perennial climbing shrub 10–20 feet long that is found growing wild in forest zones of sub-Saharan Africa (Oluwole and Okusanya 1993). Studies have shown that the

*Corresponding author: Matthias Onyebuchi Agbo, email: matthias.agbo@unn.edu.ng

African walnut possesses some properties that are required for wound healing, such as antibacterial (Ajaiyeoba and Fadare 2006), antioxidant (Amaeze *et al*. 2011) and immuno-stimulating activities (Animashaun, Adetoro and Hughes 1994). Hence, the present research is aimed at assessing the ability of these properties to accelerate wound healing on an 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. (Nottingham, UK), while lignocaine HCl (2%, 100 mg/5 mL) was obtained from Mulberry Chemicals Pvt. Ltd. (Mumbai). Methanol and n-hexane reagents were obtained from Sigma Chemicals (St. Louis, MO, USA).

Plant Material

The cooked African walnut (*T. conophorum*) was purchased from a 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 *T. conophorum* nuts were cut into pieces and air-dried. The dried nuts were ground into powder using a Willey mill (Thomas Willey Mills, Swedesboro, NJ, USA). Three hundred grams of the pulverised 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 lyophilised by a freeze-dryer to produce powdered forms of the extract. Two separate 100 g portions of fused ointment base were mixed with 5 g and 10 g of methanol extract, on a clean white tile, and the mixture was triturated carefully ensuring homogeneous mixing to obtain 5% and 10% (w/w) methanol extract formulations, respectively. The same method was used for 5 g and 10 g of n-hexane extract to obtain 5% and 10% (w/w) n-hexane extract formulations, respectively. A 1.0 g extract formulation was applied topically onto each animal wound, and wound healing progression was 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 conducted on the methanol extract to determine the safe dose according to a 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 kept without food but had access to drinking water. The mice were further starved for 3–4 h after dosing. The animals were then observed for mortality for 24 h after treatment. In the absence of death, 3 mice were

treated with 1.6, 2.9 and 5.0 g/kg body weight of the extract. The acute toxicity of median lethal dose (LD_{50}) was calculated and indicated that *T. conophorum* causes no lethality at all.

Ointment Formulation and Topical Application

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

Group 1: vehicle control; pure ointment was applied
Group 2: 5%, w/w, n-hexane extract ointment was applied
Group 3: 10% w/w, n-hexane extract ointment was applied
Group 4: 5%, w/w, methanol extract ointment was applied
Group 5: 10%, w/w, methanol extract ointment was applied
Group 6: 2%, w/w, gentamicin ointment was applied (positive control).

The animals were treated topically by applying 1.0 g of the extract formulations on the wounds twice daily. The wounds were observed daily until complete wound-healing enclosure occurred. The experiment was carried out under aseptic conditions.

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 six groups of five rats each. Rats with body weights between 180–200 g were selected for the experiment. The animals were maintained on a standard pellet diet and tap water. An approved animal ethics for use and care of laboratory animals were obtained from the local animal ethics committee of our institution. The animals received treatments topically, with each group receiving the appropriate formulation.

Excision Wound Model

Wound infliction on the backs of the animals was performed by removing hairs using a shaving machine. The wounds were induced under anaesthesia, having injected 1 mL lignocaine HCl (2%, 100 mg/5 mL) subcutaneously around the area under investigation. The excision wound was created according to the previously reported method (Odoh and Ezugwu 2007) with slight modification. The 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 the ear) using a round seal 2.5 cm in diameter. The skin of the impressed area was excised to the full thickness to obtain a wound area between 200–350 mm^2 in diameter and 2 mm deep. Haemostasis was achieved by blotting the wound with a 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

Assessment of wound contraction was done by tracing the wound area on a transparent graph paper. Evaluation of the wound surface area was performed on day 0, 4, 8, 12, 16 and 18, and this was used to determine the wound contraction assuming 100% wound

size on day 0. The period of re-epithelialisation was also observed for each of the treatment groups.

The percentage of wound contraction was determined using the following equation (Singhal, Gupta and Bhat 2011):

$$\% \text{ wound contraction} = \frac{\text{Wound area on day 0 - wound area on Nth day}}{\text{Wound area on day 0}} \times 100$$

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

Statistical Analysis

The data were expressed as the mean±SEM of at least triplicate determinations (n=3). To demonstrate the statistical significance of data, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's post hoc test, using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Differences between test and control treatments were considered significant at $p \leq 0.05$.

RESULTS

Several concentrations ranging from 5%–10% (w/w) of the n-hexane and methanol extracts of *T. conophorum* were evaluated for their wound healing activity in rats in vivo. It was observed that the wound healing ability of the extracts was 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

As shown in Table 1, in the preliminary photochemical screening of the *T. conophorum* extracts, the presence of alkaloids, saponins, glycosides, flavonoids and tannins were detected in the methanol extract, while steroids, terpenoids and triterpenes were found to be present in the n-hexane extract.

Table 1: Phyto-constituents of *T. conophorum* extracts.

Constituents	Methanol extract	n-hexane extract
Saponins	+	–
Alkaloids	+	–
Glycosides	–	–
Tannins	+	–
Flavonoids	+	–
Terpenoids	–	+
Steroids	–	+
Triterpenes	–	+

Note: + = present, – = absent

Acute Toxicity

The acute toxicity test of the methanol extract determined that the extract was safe, with a LD_{50} of above 5.0 g/kg body weight.

Wound Healing Ability

The wound healing activity of *T. conophorum* nut extracts is shown in Figure 1. 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 (Fig. 2). There was a significant reduction in wound area from the fourth day of treatment, with a much faster closure rate than that of control rats (Fig. 3). Significant wound contraction was observed in the test group from day 8 in the 10% n-hexane extract group (p=0.05), with the rest groups showing significant contraction from day 12. Additionally, the 10% n-hexane and methanol groups were the only groups that showed significant wound contraction on day 18 (p=0.05).

Re-epithelialisation of Wounds

The re-epithelialisation period for all the treatment groups is shown in Figure 4. The standard group had the shortest period of epithelialisation (19 days), while the negative control group had the longest period (28 days). The re-epithelialisation period of the test group rats was longer than that of the positive group rats (21–25 days).

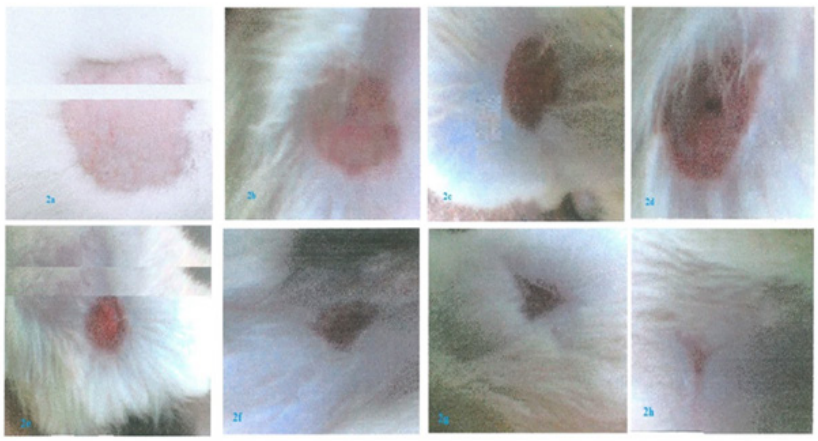

Fig. 1: Wound healing activity of different extracts of the *T. conophorum* nut: a = wound induction before treatment; b = day 11 treatment with pure ointment; c = day 15 treatment with pure ointment; d = day 11 treatment with 5% pure ointment; e = day 15 treatment with 5% methanol extract ointment; f = day 18 treatment with 10% n-hexane extract; g = day 18-treatment with 10% methanol extract ointment; h = day 15 treatment with 2% gentamicin ointment.

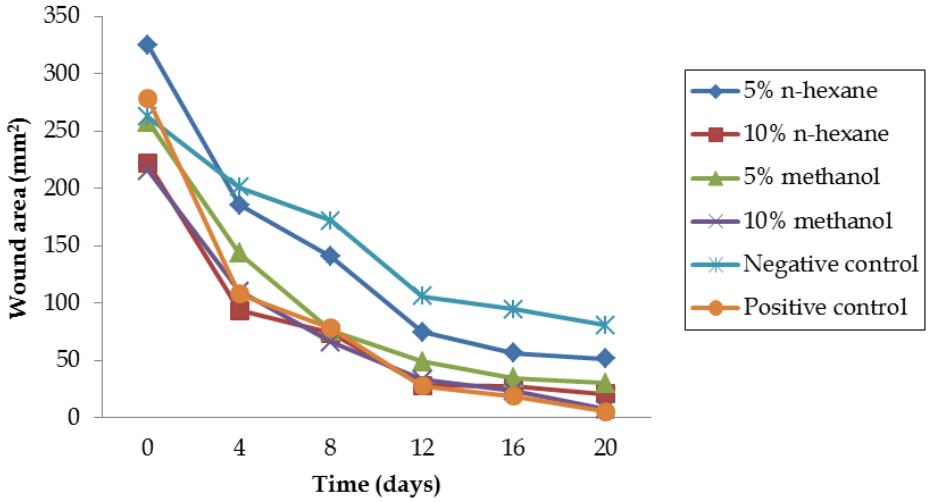

Fig. 2: Wound contraction in experimental animals.

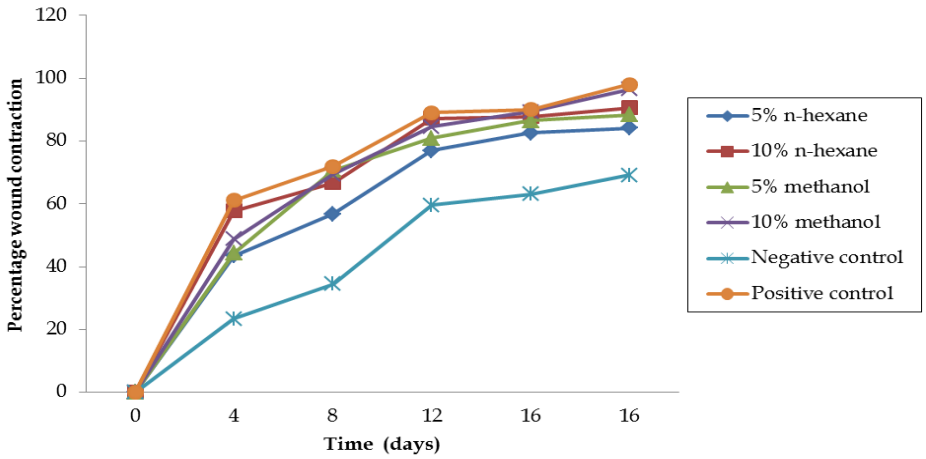

Fig. 3: Percentage wound contraction in treated animals.

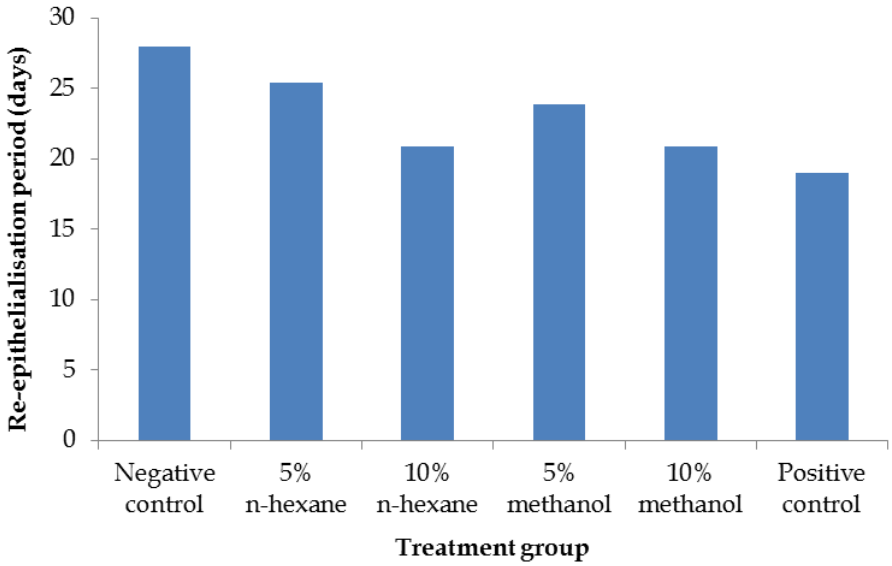

Fig. 4: Re-epithelialisation period in all treatment groups.

DISCUSSION

The process of wound healing is aimed at restoring the damaged cellular structures and tissues close to their original state (Abdulla *et al*. 2010). This helps in the restoration of the disrupted anatomical continuity and functional structure of the skin. The healing process of wound involves four stages viz: coagulation of the blood vessels, inflammation and debridement of the wound, re-epithelialisation and colleen deposition and remodelling (Phillips, Whitehe and Kinghton 1991). Wounds require treatment to either shorten the time for healing or to minimise the undesired consequences (Rajinder *et al*. 2008). Treatment requires that attention be focused on agents that can suppress wound progression like corticosteroids, antineoplastics or non-steroidal antiinflammatory agents and in disease conditions.

The *T. conophorum* nut extracts exhibited significant wound healing activity compared to the negative control in an excision wound model. It was observed that the wound contracting ability of the extract ointment-treated groups was significant (p=0.05) from day 4 onwards. The wound closure of the extract ointment-treated group decreased as the treatment days increased, which was determined by the occurrence of significant wound contraction, as shown in Figure 3.

The proliferative phase of wound healing, which involves wound contraction, occurs through the centripetal movement of the tissues surrounding the wound. This process is mediated by myofibroblasts, which establish a grip on the wound margins and contract themselves in a manner similar to that of smooth muscle cells (Midwood, William and Schwarzbauer 2004). Wound shrinking process rely on a number of factors i.e. the reparative abilities and general health state of the tissues, and the type and extent of the damage to the tissues (Pereira *et al*. 2012). In both excision and burn wounds, the wound healing progressions are monitored; thus, a comparison could be made between the two types of wounds in this present study (Pawar, Chaurasiya and Jain 2013). The observed

wound contraction in the treated groups could be attributed to the enhanced activity of fibroblast in *T. conophorum* nut extracts.

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

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

The wound healing activity observed in the extracts could be attributed to the presence of secondary metabolites in the nuts. In the preliminary photochemical analysis of the methanol extract from *T. conophorum*, we found that it contains active constituents e.g. flavanoid and tannins that are needed by the body for wound healing. *T. conophorum* extracts may have exerted their wound healing activity due to the presence of flavonoids which protect tissues from oxidative damage (Saurez, Herreta and Marhuenda 1993).

The 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 the regeneration and organisation of the new tissue (Leite *et al*. 2002).

The presence of triterpenoids and saponins in the extracts could be attributed for the contraction of wound and accelerated rate of epithelialisation. The radical-scavenging property of flavonoids enhances its antioxidant enzyme levels in granuloma tissue (Shenoy *et al*. 2009).

The extraction of total alkaloids and tannins from *T. conophorum* (Nigerian walnut) seeds have been reported (Ayoola, Onawumi and Faboya 2011). Additionally, the phytochemical analysis of the aqueous extracts of the seeds of *T. conophorum* has been investigated (Uche, Obianime and Aprioku 2010). The results 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 exhibit similar relative polarity.

CONCLUSION

The present study has shown that the extracts of *T. conophorum* had significant wound healing activity which could be attributed to the secondary metabolites like flavonoids with reported antioxidant and immuno-stimulating activities.

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