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Original research

Annona muricata leaves accelerate wound healing in rats via involvement of Hsp70 and antioxidant defence

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HIGHLIGHTS

- This study substantiated the traditional use of *Annona muricata* upon wound injury.
- *A. muricata* leaves accelerated various stages of wound healing.
- Wound healing effects were accompanied with epithelialization and collagen synthesis.
- Immunohistochemical analysis showed the up-regulation of HSP70 protein.
- The protective mechanism was through suppression of the oxidative stress.

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ABSTRACT

Introduction: *Annona muricata*, a member of the Annonaceae family, is commonly known as soursop and graviola. The leaves of this tropical fruit tree are widely used in folk medicine against skin diseases and abscesses, however there is no scientific evidence justifying the use of *A. muricata* leaves. The aim of the present study is to evaluate the wound healing potential of ethyl acetate extract of *A. muricata* leaves (EEAM) towards excisional wound models in rats.

Methods: *Sprague Dawley* rats (24) were randomly divided into four groups, viz. (A) vehicle control, (B) low dose of EEAM (5% w/w), (C) high dose of EEAM (10% w/w) and (D) positive control with excisional wound created on the neck area. Wounds were topically dressed twice a day for 15 days. On the 15th day, animals were sacrificed and then processed for immunohistochemical and histological evaluations, including Hematoxylin & Eosin and Masson Trichrome stainings. The activity of antioxidants, namely catalase, glutathione peroxidase and superoxide dismutase, and malondialdehyde (MDA) was measured in wound tissue homogenate.

Results: Macroscopic and microscopic analysis of wounds demonstrated a significant wound healing activity shown by EEAM at two doses. Treatment of wounds with ointment containing EEAM caused significant surge in antioxidants activities and decrease in the MDA level of wound tissues compared with vehicle control. The immunohistochemical evaluation revealed conspicuous up-regulation of Hsp70 in treated wounds with EEAM, suggesting the anti-inflammatory effect of EEAM.

Conclusion: EEAM exhibited a promising wound healing potential towards excisional wound models in rats.

1. Introduction

A wound is generally defined as a loss or damage of cells which breaks the anatomic or functional continuity of the skin [1]. A dynamic process of wound healing requires an elaborate biological

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cascade of cellular, biochemical, molecular and physiological events which mainly consists of four phases, namely coagulation, inflammation, proliferation and maturation or tissue remodeling [2,3]. The constant cell-matrix and cell–cell interactions during the process of wound healing makes these phases fairly integrated and overlapped [4]. The result of this complex phenomenon leads to connective tissue recovery and fibrous scar reconstruction which result in mending of anatomical continuity and functional role of the living tissue [5].

A variety of cells and tissues with collaborative efforts are involved in the process of healing. Coagulation and inflammation together as phase 1 of the healing process requires the neutrophils migration at wound margin, developing to the fibrin clot. In phase 2 or the proliferation phase, replacement of neutrophils by macrophages is an important step [6]. In addition, the incision space is filled by the invasion of granulation tissue. In the maturation or tissue remodeling as the last step of healing, the collagen accumulation and fibroblast proliferation cause an elevation in the tensile strength of the skin associated with conspicuous decrease in edema and leukocyte infiltration [6,7]. The healing process is only finished after complete mending of disrupted surface by collagen [8]. In recent years, there has been a noteworthy surge of interest in using natural remedies with more efficient healing potential against different dermatological and skin disorders, including burns, cuts and wounds [9]. Medicinal plants have provided more affordable wound healing products with higher safety from hypersensitive reactions compared to the synthetic pharmaceutical agents [10,11].

The popular fruit tree of *Annona muricata* L. (Annonaceae) commonly known as graviola or soursop is commercially cultured in Central and South America and tropical countries [12]. The edible fruit of this small tropical tree is extensively used to produce candy, juice and sherbets [13]. In addition, the importance of this plant is also contributed to its various applications in folk medicine [14]. All parts of this plant, including the bark, fruit-seeds, leaves and root are used in natural medicines in the tropics [15]. Traditionally, the leaves are used for cystitis, diabetes, headaches, hypertension, insomnia, liver problems and as an antidysenteric, anti-inflammatory and antispasmodic [16]. The cooked leaves are topically applied against abscesses [16]. In tropical Africa, including Nigeria, leaves are traditionally used against skin diseases [15]. However, the wound healing potential of the *A. muricata* leaves has not been replicated in an experimental animal study as of yet. Therefore, the present study was designed to examine the efficacy of ethyl acetate extract of *A. muricata* leaves (EEAM) towards excisional wound healing on rats.

2. Materials and methods

2.1. Materials

A blank placebo (an aqueous semisolid cream) and intrasite gel (standard wound dressing drug, Smith & Nephew Ltd., UK) were purchased from the pharmacy of University of Malaya Medical Centre. The intrasite gel used as positive control contains modified carboxymethyl cellulose polymer (2.3%), propylene glycol (20%) and water, and promotes natural debridement by rehydrating necrotic tissue. The leaves of *A. muricata* were collected from Ipoh, Malaysia, in March 2013, and was authenticated by Dr. Yong Kien Thai at Institute of Biological Sciences, Faculty of Science Building, University of Malaya. The voucher specimen of the same has been deposited at the herbarium of the University of Malaya (voucher specimen No. KLU47978).

2.2. Preparation of the leaves extract

The leaves (1 kg) were cut into small pieces, washed with distilled water and dried in the oven at 45 °C for 5 days. After grinding the leaves, the leaf powder was soaked in ethyl acetate (1500 ml, three times) in conical flasks for 3 days at 25 °C. The residue was removed by filtration using filter papers (Whatman No. 1). Afterwards, the extract was concentrated by recovering the solvent using a rotator evaporator (Buchi, Flawil, Switzerland). After drying the extract in the vacuum oven at 40 °C, the semisolid mass of EEAM (15 g, ethyl acetate free) was homogeneously mixed with the placebo in two concentrations (5% and 10% w/w) and stored at 2–8 °C in the refrigerator for further evaluation of wound healing activity.

2.3. Animals

Healthy adult male *Sprague Dawley* rats weighing 180–250 g from the animal house of AEU (Animal Experimental Unit, University of Malaya) were used in this study. Rodents were maintained in clean, sterile, polyvinyl cages with normal pellet diet and water *ad libitum*. They were housed under standard environmental conditions of humidity and temperature (25 ± 0.50 °C) with 12 h light/dark cycle. The animal studies were carried out in AEU with due permission from the FOM Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC, ethic No.: 2014-03-05-PHAR/R/SZM).

2.4. Excision wound model

The acute wound healing activity of EEAM in this study was tested on uninfected excisional wounds. The wounding creation was performed under general anaesthesia by using 2 ml of diethyl ether (99% purity, Sigma Chemical Co., St. Louis, MO, USA). After shaving and disinfecting the skin with alcohol (70%), a local anaesthetic injection was performed by lignocaine HCl (1 ml, 2%, 20 mg/ml). After marking an oval wound, a uniform wound (approx. 500 mm^2) with 2 mm depth was excised from the nape of the dorsal neck of each rat using a pair of surgical scissors aseptically (Fig. 1). The neck area of wound was chosen to avoid any unwanted biting and stretching from the rats. Any damage to the muscle layer was carefully avoided and all the procedure was performed with the constant tension of the skin. The forceps and scissors were cleansed with alcohol (70%) after each use.

2.5. Grouping, topical treatment and sampling

Rats were grouped into four groups, viz. (A) vehicle control, (B) low dose (5% w/w), (C) high dose (10% w/w) and (D) positive control group. Each group consisted of six animals, and treatments were started 24 h after the wounding procedure. The wounds of the vehicle control group (group A) were dressed topically twice daily with blank placebo (0.2 ml). With the same procedure, the positive control group (group D) was treated with intrasite gel (0.2 ml) and two doses of EEAM (5% and 10% w/w, 0.2 ml) were used for the treatment of the low dose (group B) and high dose (group C) groups, respectively.

2.6. Determination of the wound closure percentage

The wound closure area (mm^2) of each rat was measured by tracing the wound on days 5, 10 and 15 using a permanent marker and transparent papers under light anaesthesia with ketamine and xylazine. At these days, the percent wound healing values were determined for each group by calculating the percentage of wound reduction from the original wound.