Original research

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

Soheil Zorofchian Moghadamtousi a, Elham Rouhollahi b, Maryam Hajrezaie a, Hamed Karimian c, Mahmood Ameen Abdulla d, Habsah Abdul Kadir a, * b

a Biomolecular Research Group, Biochemistry Program, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
b Department of Pharmacology, Faculty of Medicine, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
c Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
d Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

**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.

**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.

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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...
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 carbomethyl 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 homogeneous 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 ± 0.5 °C) with a 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 uninjured 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 mm²) 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 (mm²) 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.
buffer (2 ml, 50 mM, pH 7.0) and sodium azide (0.3 ml, 1 mM). The
enzyme solution (0.2 ml) was added to the reaction mixture
following incubation for 5 min at 25 °C. Then, the reaction
was started by adding 
H2O2 (0.2 ml, 0.25 mM). The optical density
presenting the GPx activity was determined at 340 nm. The result is
expressed as nmol of NADPH oxidized/min/mg protein. The level of
catalase (CAT) and superoxide dismutase (SOD) in tissue homoge-
nate was measured using commercial kits (Cayman, Ann Arbor, MI,
USA) according to the manufacturer's instructions.

2.10. Lipid peroxidation

Determination of Lipid peroxidation in wound tissue homoge-
nate was performed using the TBA reaction as previously described
in detail [19]. TBA reaction measures the level of malondialdehyde
(MDA) as a product of lipid peroxidation. The result is expressed as
nmol of MDA formed/mg protein.

2.11. Statistical analysis

All values were reported as means ± SEM of n animals per group.
Statistical evaluation of the data was performed using one-way
analysis of variance (ANOVA) followed by Tukey’s test. Differ-
ences were considered statistically significant when P < 0.05.

3. Results and discussion

3.1. Effect of EEAM on wound closure

As it is illustrated from Fig. 2, on day 5 of treatments, each group
revealed different rates of wound contraction, however intrasite gel
and EEAM (only at 10% concentration) caused significant reduction
in the wound area, compared to the vehicle control (Fig. 2). After
10 days of topical treatments with EEAM (5% and 10%) and intrasite
gel, all three groups showed significant elevation in wound con-
traction, compared to the vehicle control. Administration of
EEAM (5% and 10%) and intrasite gel after 15 days caused 69%, 77%
and 81% wound closure, respectively (Fig. 3).

3.2. Histopathological effect of EEAM in wound tissues

Histopathological survey of wound tissues after the surgery on
day 15 was carried out using H & E and Masson Trichrome stainings.
As illustrated in Fig. 4, tissue sections stained with H & E demon-
strated that wound areas in treated rats with EEAM (5% and 10%)
and intrasite gel were conspicuously smaller than those treated
with the blank placebo. On day 15, EEAM treated rats at both
concentrations, especially 10%, demonstrated very close profiles
when compared to the positive control group. Dermis maturation
and organization of collagen fibers were examined by the Masson
Trichrome staining (Fig. 5). In blank placebo treated wounds,
collagen fibers were characterized by poor orientation and disor-
ganization. The scattered collections of inflammatory cells,
including macrophages and neutrophils, with disorganized-
oriented collagen fibers displayed the immature tissue granula-
tion in vehicle control group. However, EEAM treatment markedly
stimulated and elevated the deposition of collagen fibers which
were comparable with the positive control.

3.3. EEAM induced up-regulation of Hsp70 in wound tissues

In our study, upon treatment of wounds with EEAM (5% and
10%), protein expression of Hsp70 was markedly increased in the
wound tissues, which were comparable with Hsp70 up-regulation
in the positive control group (Fig. 6).
3.4. Effect of EEAM on enzymatic activities in wound tissues

Changes in the SOD activity of the wound tissue of rats after administration of EEAM and intrasite gel are shown in Table 1. Topical treatment with EEAM (5% and 10%) and intrasite gel led to significant elevated activity of the enzyme in the wound tissue compared to the vehicle control. The significant increase in SOD activity after EEAM (5% and 10%) treatment appears to be a

![Fig. 2. Gross appearance of the healing of excisional wound in rats at day 15. The rats were topically dressed (0.2 ml) with (A) blank placebo, EEAM at two doses (B) 5%, (C) 10% w/w and (D) intrasite gel. EEAM, ethyl acetate extract of A. muricata leaves.](image)

![Fig. 3. Effect of topical treatments on percentage of wound closure in four groups. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Data are reported as means ± SEM of six animals per group. A value of P < 0.05 was considered significant. EEAM, ethyl acetate extract of A. muricata leaves.](image)
A study on wound healing activity of the Annonaceae family, including species of the Annona family, has shown that they contain major bioactive compounds in these species [23]. Previous studies have demonstrated that treatment of wound area with EEAM extract at high dose of EEAM and (D) positive control. The white arrows illustrate the wound area for each group. D, dermis; E, epidermis; EEAM, ethyl acetate extract of A. muricata leaves; GT, granulation tissue; S: scar width. Scale bar: 500 μm.

Fig. 4. Histological analysis (H & E) of wound tissues on day 15 after operation, from four groups of rats. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. The white arrows illustrate the wound area for each group. D, dermis; E, epidermis; EEAM, ethyl acetate extract of A. muricata leaves; GT, granulation tissue; S: scar width. Scale bar: 500 μm.

3.5. Effect of EEAM on MDA level in wound tissues

Levels of MDA in the topically treated rats with EEAM (5% and 10%) and intrasite gel revealed a significant reduction compared to the vehicle control group (Fig. 7). This result strongly suggests that EEAM treatment at both doses markedly attenuated the lipid peroxidation in the wound site of rats.

4. Discussion

A. muricata were previously found to possess chemical constituents of different alkaloids and essential oils [20–22]. Nonetheless, species of the Annonaceae family, including A. muricata are well known to have a variety of acetogenins compounds that act as major bioactive compounds in these species [23]. Previous studies have shown that Annona species have promising wound healing capability. A study on wound healing activity of A. muricata stem bark revealed conspicuous decrease in the wound area of rats after topical treatment with the alcoholic extract of stem bark [24]. In another study, Annona squamosa leaves demonstrated a promising wound healing effect against streptozotocin-induced diabetic rats [25]. Wound healing studies generally evaluate two simple and reproducible wound models, namely incisional and excisional [26]. Considering the promising wound healing activity of the Annona species, in this study, full thickness excisional wound model was applied to macroscopically and histologically investigate the wound healing potential of EEAM on rats.

The untreated wounds in vehicle control group were characterized with stiff and intact dark brown scabs (Fig. 2). The findings in the current study demonstrated that topical administration of EEAM noticeably accelerated the wound healing process in rats. New thin epidermis formed in EEAM (5% and 10%) treated wounds which provided the protection to the wounds from further injuries by covering the entire wound area (Fig. 4). Epidermis layer of wounds from EEAM (5% and 10%) treated rats elicited a well-advanced organization of granulation tissue and ongoing formation of new blood vessels which were comparable with positive control group. The tissue sections from the EEAM (5% and 10%) treated wounds revealed well-organized accumulation of collagen fibers with fewer inflammatory cells compared to the vehicle control group (Fig. 5). The histological analyses of rats demonstrated that treatment of wound area with EEAM extract at both doses markedly accelerated the original tissue regeneration.

Heat shock proteins (Hsps) are crucial factors for the wound healing process due to their role in cell proliferation, collagen synthesis, modulation of inflammation and wound debris clearance. Despite the abundance of Hsps in cells, their expressions are modulated at the basic levels under normal physiological conditions [27]. Under the wound healing stress, Hsp expression dramatically elevates to attenuate the inflammatory responses and to accelerate the wound healing process [28]. Therefore, any perturbation in the expression and function of Hsps in response to various cellular stress may lead to a wide variety of wound healing complexities [29]. As the abundant inducible Hsp in the wound bed,
Hsp70 is effectively responsible for the cell survival and protein homeostasis within the healing wound [30]. The immunohistochemistry analysis in our study revealed that the up-regulation of Hsp70 by EEAM was comparable with the effect of intrasite gel. This result strongly implies that EEAM induced the protein expression of Hsp70 in the wound tissue which led to amelioration of the wound healing process.

The process of wound healing is accompanied with skin ischemia, which promotes the generation of reactive oxygen species by activated leukocytes in the tissue site. The more release of oxygen-derived free radicals through the positive feedback attracts more leukocytes and amplifies the oxidative damages in the wound tissue [31]. Under normal conditions, body homeostasis balances the level of free radicals using the endogenous antioxidant capacity of the human body. However, when this level exceeds the normal capacity of antioxidants to balance, highly activated radicals will cause different structural changes and further reversible or irreversible cell injuries [32,33]. Antioxidant defence systems of cells contain a variety of enzymatic and non-enzymatic scavengers. The enzymatic antioxidants of cells, including CAT, GPx, glutathione reductase (GR), glutathione-s-transferase (GST), SOD play a critical role in the attenuation of oxidative stress induced by reactive oxygen species [34]. The first defensive mechanism against reactive oxygen species is provided by SOD, which attenuates oxidative stress through dismutation of O$_2^-$. CAT enzyme has an important role in converting the endogenous H$_2$O$_2$ to water and oxygen [35].

The accumulation of H$_2$O$_2$ in cells results in the generation of highly reactive free hydroxyl radical (OH$^-$) through Fenton reaction, which has an important devastating role in oxidative damages [36,37]. Another important antioxidant enzyme, GPx, degrades lipid peroxides to hydroxyl lipids and waters through conversion of glutathione to glutathione disulfide [38].

The excessive production of reactive oxygen species and oxidative stress in the wound site is known to cause lipid peroxidation in the respective tissue [39,40]. Lipid peroxidation of organelle and cellular membranes, being one of the destructive effects of oxygen radicals, is responsible for the defect in endothelial cells, fibroblast and collagen metabolism and keratinocyte capillary permeability. In addition, the elevated lipid peroxidation in the wound tissue may have been a contributing factor in the impairment of vascular endothelial growth factor (VEGF) expression and subsequently deficiency of the wound healing process [41]. As the main oxidation product of peroxidized polyunsaturated fatty acids, MDA is a critical biomarker for lipid peroxidation [42].

Previous in vitro and in vivo studies also reported the antioxidant activity of Annona muricata leaves [43,44]. Among different Annona species, leaves of A. muricata were found to have the highest antioxidant activity assessed by DPPH radical scavenging activity, FRAP (Ferric reducing antioxidant property) and HRSA (hydroxyl scavenging activity) techniques [44]. An in vivo study also showed that ethyl acetate extract of A. muricata leaves caused an increase in the activity of CAT, glutathione and SOD on gastric cells.
of ethanol-treated rats [45]. Our findings in the present study strongly suggested that topical administration of EEAM on wounds conspicuously increased the antioxidant capacity of the wound tissue leading to hastening of the wound healing process.

5. Conclusion

In conclusion, these findings showed that EEAM accelerates various stages of wound healing, including wound contraction, epithelialization and collagen synthesis. In addition, it also decreased the oxidative and inflammatory stresses in the wound area. Treated wounds with EEAM displayed organized generation of collagen fibers with reduced number of inflammatory cells.

However, further study with bio-assay guided approach is still required to establish the principle bioactive compound responsible for the wound healing effect of EEAM.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>CAT (nM/min/ml)</th>
<th>GPx (nM/min/mg)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>85.45 ± 3.78</td>
<td>16.19 ± 1.34</td>
<td>14.25 ± 1.15</td>
</tr>
<tr>
<td>B</td>
<td>115.7 ± 1.29</td>
<td>23.19 ± 0.94</td>
<td>19.25 ± 0.73</td>
</tr>
<tr>
<td>C</td>
<td>131.29 ± 2.44</td>
<td>29.25 ± 1.13</td>
<td>27.25 ± 1.13</td>
</tr>
<tr>
<td>D</td>
<td>146.11 ± 4.17</td>
<td>31.67 ± 2.02</td>
<td>36.78 ± 2.6</td>
</tr>
</tbody>
</table>

Antioxidant enzymes include CAT, SOD and GPx from four groups of rats namely, (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Data are reported as means ± SEM of six animals per group. A value of P < 0.05 was considered significant. CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase.

Fig. 6. Immunohistochemical analysis of Hsp70 protein expression from four groups of rats. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Immunohistochemistry staining showed up-regulation of Hsp70 in groups B–D. The red arrows depict Hsp70 protein accumulation in gastric tissue. EEAM, ethyl acetate extract of A. muricata leaves. Scale bar: 10 μm.

Fig. 7. Effect of ointment treatments on MDA level in wound tissue homogenate from four groups. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Data are reported as means ± SEM of six animals per group. A value of P < 0.05 was considered significant. EEAM, ethyl acetate extract of A. muricata leaves.

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Ethical approval

The animal studies were carried out in AEU (Animal Experimental Unit, University of Malaya) with due permission from the FOM Institutional Animal Care and Use Committee, University of Malaya (FOMIAUC, ethic No.: 2014-03-05-PHAR/R/SZM).

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Author contribution

Soheil Zorofchian Moghadamtousi, Mahmood Ameen Abdulla and Habsab Abdal Kadir conceived and designed the study. Soheil Zorofchian Moghadamtousi and Elham Rousholahi performed the experiments. Maryam Hajrezaie and Hamed Karimian analysed the data. Mahmood Ameen Abdulla and Habsah Abdul Kadir contributed reagents/materials/analysis tools. Soheil Zorofchian Moghadamtousi wrote the manuscript.

Disclosure

The authors declare that they have no conflict of interests.

Guarantor

Mahmood Abdulla Abdulla and Habsab Abdal Kadir accept full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

References