Wound healing potency of *Terminalia catappa* in mice (*Mus musculus*)

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Abstract
Leaves of *Terminalia catappa* and its phytoconstituents are well known as a natural promising medicine especially for wound healing agents. The purpose of the current research was to evaluate the wound healing potency of ethanolic and water extract of *T. catappa* either green or brown leaves in mice (*Mus musculus*) model. A group of mice were used and randomly divided into control, vaseline, povidone iodine groups and treatment groups consisting ethanolic extract either green (GLE) or brown leaves (BLE), and water extract green (GLW) and brown (BLW) leaves of *T. catappa* as ointment. All mice were prepared for excision and treated with simple ointment once a day until the wound was closure. The percentage of wound closure was recorded every three days, while total DNA and hydroxyproline content were determined at day 12. Qualitative phytochemical assay was performed to determine the phytoconstituent in the leaves extract. The results found that mice treated with GLE and GLW had better wound healing at the day 12, confirming that the wound healing activity was found to be better than control and Povidone-iodine group. Furthermore, hydroxyproline content were also found to be higher in all treated groups than control group. Present finding suggested that *T. catappa* leaves extract which contains important phytochemicals shows a good wound healing activity and can be used as alternative medicine for wound healing.

Keywords: mice, phytochemicals, *Terminalia catappa*, wound healing

INTRODUCTION
The Indian almond tree or known as *Terminalia catappa* is a native to Southeast Asia and widely distributed in both tropical and sub-tropical regions. The *T. catappa* is an attractive, long-lived tree well suited to ornamental and amenity plantings. The leaves of *T. catappa* are useful as a folk medicine such as antidiabetic, analgesic, antiulcer, hyper glycaemia, and liver-related diseases (Anand et al. 2015, Behl and Kotwani 2017, Pinheiro Silva et al. 2015). Previously reported also obtained that the leaf of *T. catappa* has an antibacterial properties (Allyn et al. 2018, Nugroho et al. 2017) and it contains benefit phytochemicals such as saponin, triterpenoid, quinone, phenolic, tannin, and flavonoid (Nugroho et al. 2016), that may be useful for wound healing.

Wound healing is a natural repairing process of the damage tissue that consists of four biochemical events: blood coagulation, inflammation which is immediately begins after injury, cell proliferation, lesion contraction and remodeling (Ghuman et al. 2019, Hermes et al. 2013). In the proliferation event, a forming of granulation tissue is built by fibroblast and the angiogenesis process (Dwivedi et al. 2017). According to Lodhi and Singhai (2013), a successful of wound healing process depends on the several factors such as blood cells and parenchymal cells component in a time frame, extracellular matrix molecules, soluble and biochemical mediators.

Further, determining the DNA and hydroxyproline content at wound healing tissue is a well-established procedure for monitoring normal cell proliferation (Nordin et al. 2018).The enhance in the DNA and hydroxyproline content is a result of increased cell division (Ahmad et al. 2017). Moreover, the increase of DNA content can be stimulated by Platelet-derived growth factor (PDGF) which also triggered chemotaxis of fibroblasts and production of collagen, glycosaminoglycan, and collagenase (Lynch et al. 1987). Meanwhile, previous study also revealed that there was a high content of hydroxyproline due to the
effects of *Rhus coriaria* extract on Sprague Dawley rats excision wound model (Amin 2018).

Though some wound model studies have been done by using several medicinal plant extracts, study of *T. catappa* which related to wound healing activity in mice model is limited. Thus, present project was aimed to determine the wound healing properties of *T. catappa* leaves by measuring the percentage of wound enclosure, DNA and hydroxyproline content. Some preliminary qualitatively phytochemical test in the crude extract of *T. catappa* leaves was also evaluated to detect presence possible phytochemical such as: flavonoid, quinon, triterpenoid, steroid, saponin, tannin, phenolic, and alkaloid.

**MATERIALS AND METHODS**

Plant Materials and Ethanol Extract Preparation

The *T. catappa* green and brown leaves were collected from around Mulawarman University, Samarinda, East Kalimantan, Indonesia. To eliminate extraneous matter, the leaves were cleaned with distilled water and immediately dried in an oven at 40°C for 12h. The leaves were later cut and ground to make them powdery using a mill and the powder extracted using ethanol 95% for 2 days (100 g L\(^{-1}\)). The obtained extracts were filtered and put in the dark bottle and keep in 4°C until used.

Preliminary Test for Phytochemicals

To detect the presence for flavonoid in either green or brown leaves, a 2 mL leaves extract was added with concentrated hydrochloric acid and magnesium ribbon. The pink-red color indicated the presence of flavonoids. Meanwhile, quinon test was performed by adding 1 mL of leaves extract with 1 mL of concentrated sulfuric acid. A red color indicated the presence of quinon. The presence of triterpenoid and steroid in the *T. catappa* leaves extract can be detected by Liebermann-Burchard methods. Respectively, a 2 mL of leaves extract was added with 1 mL chloroform, a few drops of acetic anhydride and concentrated sulphuric acid. The presence of triterpenoid was shown by a red or brown color, while steroid by a blue or green color. The occurrence of saponin in the leaves extract can be qualitatively monitored by mixing 1 mL of extract and 5 mL distilled water. The mixture was shaken vigorously to develop the froth. The occurrence of stable froth for 15 minutes indicated the presence of saponin. The presence of tannin and phenolic was determined by adding leaves extract with 2 mL of 2% solution of FeCl\(_3\). A blue-green or black color results revealed the presence of phenols and tannins. To screen possible alkaloid content in the leaves extract, Dragendorff Test was done by mixing 2 mL of the leaves extract with 1 mL of Dragendorff reagent. The orange or orange-reddish-brown precipitate stated a positive result for alkaloid presence.

Acclimated Animals

In total of thirty-five mice (20–25g, male, 3 months old) were purchased from the local animal husbandry unit in Samarinda, Indonesia. Mice were reared and acclimated in standard housing conditions at room temperature (±25°C) with a light/dark cycle of 12/12h for 3 days. Feed and water were given ad libitum methods. After acclimatization, mice were randomly divided into seven groups, namely Control group without treatment as negative control, vaseline group as placebo group which treated with vaseline (base ointment), Povide iodine group as positive control, treatment groups consisting base ointment with 60% of either ethanol or water extract of green leaves of *T. catappa*, and another treatment groups with 60% of either ethanol or water extract of brown leaves of *T. catappa*. Each group consisted of five mice as replication.

Ointment Preparation

The ointment was made by adding vaseline with 60% of wither green or brown leaves extract of *T. catappa*. The mixture was stirred using spatula until homogeneous. The ointment was stored in the freezer and ready to be used in wound healing treatment.

Treatment Procedure

Before simple ointment treatment in each group, the hair around the back was shaved and the skin was smeared with alcohol. All mice were then anesthetized using 2% ketamine, followed by 1 cm incision with ± 1 mm depth in the back using a sterile scalpel to reach the hypodermic layer. The ointment was done by applying it to the wound in mice by using a spatula every day in the morning, from day 1 until the wound was completely closed. Each mouse was observed for wound enclosure every three days until day 15 using a callipers and tissue samples were taken on the 15th day. DNA was isolated with a commercial extraction kit (Genomic DNA Extraction Kit D1700; Solarbio, Beijing). Each total amount of DNA from each tissue sample was calculated in triplicates performed with Qubit 2.0 fluorometer (Invitrogen, USA).
The wound healing is natural process to repair an injury or damage tissue. Current result revealed that the percentage of wound enclosure in mice, treated topically with GLE was from 12% on day 3 to 100% on day 12, while GLW was 5.66% to 100% from day 3 to day 12, respectively. Meanwhile, mice in povide iodine, BLE and BLW group showed completely wound enclosure at the day 15. On the other hand, the complete wound enclosure of mice in the control and vaseline group was found on the day 15 (Table 2 and Fig. 1). In agreement with these results, Sharath et al. (2010) revealed that alcoholic extract of Bacopa monniera was found to increase wound healing enclosure by enhancing tensile strength, wound epithelization, and the formation of connective tissue. Another study also demonstrated that, Ceylon cinnamon is an effective plant to stimulate the enclosure of wounds in mice (Farahpour and Habibi 2012).

Several and various phytochemicals from plants extract have been claimed as a natural phytoconstituent that responsible in wound healing. The wound healing process may involve in enhancing antioxidant production, especially in the wound area (Habibipour et al. 2003) which related to the phytochemical content from T. catappa extract. The results current findings confirmed that T. catappa leaves extract promote the healing process due to phytochemical contents.

The phytochemicals from plant such as tannin an flavonoid are responsible in wound healing enclosure that act as astringent and increased rate of epithelization (Tsuchiya et al. 1996). Further, flavonoids are stated to be a health-promoting substances which has anti-inflammatory properties, having a crucial role in wound healing (Gomathi et al. 2003, Lodhi et al. 2016). Meanwhile, (Kim et al. 2011) revealed that saponin from the ginseng is also beneficial in wound healing process. Saponin has some beneficial such as hemolytic, anti-bacterial, anti-viral, anti-oxidative functions, anti-inflammatory activity which decrease edema and skin inflammation (Just et al. 1998, Kim et al. 2011, Navarro et al. 2001). In fact, saponin helps in accelerating neovascularization in burn wounds area in mice skin. Further, saponin also relates to vascular endothelial growth factor and interleukin (IL)-1β, both of them are in group of inflammatory cytokines that trigger the accumulation of macrophages at skin wound area, boosting wound healing (Kimura et al. 2006). In addition, phenolic may also involve in reducing inflammatory and cell growth activities.

**Table 1. Qualitative test for possible phytochemical in the T. catappa leaves extract**

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Green leaves</th>
<th>Brown leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinon</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Noted: (+): Present; (-): Absent

**Determination of Hydroxyproline Content**

To calculate the hydroxyproline content, on the day 12 of the post excision wound procedure, a piece of skin from the healed wound area was collected. Respectively, tissues from healed wound were dried in oven at 60-70°C until weight was constant. The constant weight of healed wound was then hydrolysed using 6 N HCl at 130°C for 4 h in tight sealed tube. The resulting hydrolysate was neutralized to pH 7.0 followed by adding Chloramine T oxidation for 20 min. The reaction was terminated by addition of 0.4M per chloric acid. To develop the colour Ehrlich reagent was added at 60°C and calculated at 557 nm using UV/Vis spectrophotometer (Jenway 6305, UK).

**Statistical Analysis**

All values are presented as mean ± standard error mean (S.E.M.). Analysis of variance (ANOVA) and Duncan multiple range test (DMRT) as post hoc were performed to find a significant different among the groups and A value of P < 0.05 was considered significant. All statistical analysis was done by using SPSS for windows 20 (SPSS, Inc., USA).

**RESULTS AND DISCUSSION**

Present study found that both green and brown ethanolic extract leaves of T. catappa contained phytochemicals such as flavonoid, quinon, triterpenoid, steroid, saponin, tannin, phenolic, and alkaloid (Table 1). Either green or brown ethanolic extract T. catappa leaves did not contain steroid. This finding is consistent with previous study stating that no steroid has been found in both leaves (Nugroho et al. 2019). However, ethanolic extract of brown leaves of T. catappa showed no alkaloid. This difference might be due to the fact that water/moisture content, terrain, geographical location, seasonal variation, soil type influence phytochemicals in plants. The wound healing is natural process to repair an injury or damage tissue. Current result revealed that the percentage of wound enclosure in mice, treated topically with GLE was from 12% on day 3 to 100% on day 12, while GLW was 5.66% to 100% from day 3 to day 12, respectively. Meanwhile, mice in povide iodine, BLE and BLW group showed completely wound enclosure at the day 15. On the other hand, the complete wound enclosure of mice in the control and vaseline group was found on the day 15 (Table 2 and Fig. 1). In agreement with these results, Sharath et al. (2010) revealed that alcoholic extract of Bacopa monniera was found to increase wound healing enclosure by enhancing tensile strength, wound epithelization, and the formation of connective tissue. Another study also demonstrated that, Ceylon cinnamon is an effective plant to stimulate the enclosure of wounds in mice (Farahpour and Habibi 2012).

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**Table 2. The percentage of wound enclosure of mice after ointment with Terminalia catappa leaves**

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>Vaseline</th>
<th>Povide Iodine</th>
<th>GLE</th>
<th>GLW</th>
<th>BLE</th>
<th>BLW</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
</tr>
<tr>
<td>3</td>
<td>18.66±0.66*</td>
<td>17.00±1.00*</td>
<td>29.33±2.66*</td>
<td>12.00±1.30*</td>
<td>5.66±1.45*</td>
<td>9.33±2.90*</td>
<td>2.66±0.33*</td>
</tr>
<tr>
<td>6</td>
<td>24.00±2.51*</td>
<td>34.33±2.90*</td>
<td>40.66±5.23*</td>
<td>40.00±4.16*</td>
<td>48.00±1.15*</td>
<td>53.33±5.45*</td>
<td>13.33±0.33*</td>
</tr>
<tr>
<td>9</td>
<td>47.00±1.70*</td>
<td>74.33±2.90*</td>
<td>50.00±6.66*</td>
<td>52.66±1.70*</td>
<td>70.33±1.20*</td>
<td>71.33±1.80*</td>
<td>52.00±1.15*</td>
</tr>
<tr>
<td>12</td>
<td>94.66±5.33*</td>
<td>84.66±1.76*</td>
<td>95.33±3.66*</td>
<td>100.00±0.00*</td>
<td>100.00±0.00*</td>
<td>93.33±6.66*</td>
<td>93.00±1.7*</td>
</tr>
<tr>
<td>15</td>
<td>97.00±3.00*</td>
<td>96.33±3.66*</td>
<td>100.00±0.00*</td>
<td>100.00±0.00*</td>
<td>100.00±0.00*</td>
<td>100.00±0.00*</td>
<td>100.00±0.00*</td>
</tr>
</tbody>
</table>

Note: Different letter indexes superscripts (a, b, c, d) indicate significantly different means for different treatments at P<0.05. Different numerical indexes subscripts (1-6) indicate significantly different means at different times at P<0.05. GLE = Green leaves ethanolic extract, GLW = Green leaves water extract, BLE = Brown leaves ethanolic extract, BLW = Brown leaves water extract.
increase in healing activity of cutaneous wound area in mice (Song et al. 2017). Total DNA and Hydroxyproline content are parameters that generally used in wound healing study. Present research found that total DNA from new wound tissue of mice after topical application of ethanolic (0.849 µg mL⁻¹) and water (0.852 µg mL⁻¹) extract Terminalia catappa green leaves were significantly higher than other groups (Table 3). Buffoni et al. (1993) stated that the increase of DNA content is shown during the skin healing, which is followed by protein content, reaching a maximum after several days of healing periods. Meanwhile, the hydroxyproline contents in mice treated with povide iodine, ethanolic or water extract of T. catappa brown or green leaves were found to be significantly (p<0.05) higher than the control group. The enhance in hydroxyproline content was due to a process of collagen synthesis or active proliferation of fibroblast cells that responsible for synthesizing collagens.

In conclusion, present finding stated that both ethanolic and water extract of T. catappa either green or brown have important phytoconstituent that useful for wound healing in mice. The ethanolic and water extract of green leaves has higher potency as wound healing in term of wound enclosure, total DNA and hydroxyproline content. Further research need to be conducted to determine the protein content in newly wound tissue and molecular aspects such as interleukin and responsible gene for wound healing.

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