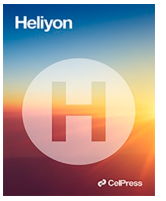
Heliyon 10 (2024) e39339

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Biochemical, immunological markers, histology and ultrastructural changes of open wound healing in rats treated with ethyl acetate extract of *Zingiber zerumbet* rhizomes

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ARTICLE INFO

Keywords:
Wound healing
Z. zerumbet
Ethyl acetate
Biochemical markers
Ultrastructure

ABSTRACT

Research on plant-based wound healing agents has been one of the current developing areas in modern biomedical science. This study aimed to assess the effects of ethyl acetate extract of *Zingiber zerumbet* rhizome (ZZRE) on open wound healing activity in Wistar rats. Ninety male Wistar rats (220–320 g) were divided into three groups treated with phosphate buffered saline (PBS) (negative control), Solcoseryl gel (positive control), and 10 % ZZRE (treatment group), respectively. Six circular full-skin thickness wounds of 6.0 mm in diameter were induced bilaterally on the dorsal surface of each rat. Six rats were sacrificed on Day-1, Day-3, Day-6, Day-10 and Day-14 respectively from each group after wound induction. All data obtained are considered statistically significant at $p < 0.05$. Macroscopic observations showed that the 10 % ZZRE treated wounds healed faster compared to other groups. The wound closure percentage showed that the wound treated with 10 % ZZRE is significantly higher ($p < 0.05$) than the PBS group on Day-6, Day-10 and Day-14. Protein levels of the 10 % ZZRE group decreased significantly at Day-1 compared to the PBS group and significantly ($p < 0.05$) higher at Day-14 compared to both control groups. The hexosamine and uronic acid levels of the 10 % ZZRE group showed a significant ($p < 0.05$) decrease on Day-14. Conversely, hydroxyproline levels showed significant ($p < 0.05$) increase starting from Day-3 until Day-14. As for the immunological markers, the level of total TGF-β1 of the 10 % ZZRE group was significantly ($p < 0.05$) higher than the PBS group on Day-14, whereas the level of IL-10 on the wound tissue of the 10 % ZZRE group was significantly ($p < 0.05$) lower than the PBS group on Day-1 but significantly ($p < 0.05$) higher on Day-10 and Day-14 compared to both control groups. Histological observation showed that the wounds treated with 10 % ZZRE infiltrated with lesser inflammatory cells while collagen deposition was denser as compared to both control groups. Based on the result obtained, it is clearly proven that treatment of 10 % ZZRE on open wound healing in rats, showed that the extract was effective in healing the wound and accelerated the healing process. Therefore, the 10 % ZZRE tested has the potential to be developed as an alternative wound healing agent in the future.

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https://doi.org/10.1016/j.heliyon.2024.e39339
Received 20 February 2024; Received in revised form 11 October 2024; Accepted 11 October 2024
Available online 16 October 2024

1. Introduction

A wound is a physical injury that leads to the damaging of skin surfaces. Wounds have always been a main concern of physicians and patients as the quality of life would be affected. In fact, wound healing is categorized as one of the most intricate processes in humans and plays an essential role in returning the normal status of skin functions [1,2]. Wound healing processes can be divided into four phases: namely hemostasis, inflammatory, proliferative and remodeling. The hemostasis phase starts immediately after skin injury and is characterized by vasoconstriction and the formation of fibrin clots. The inflammatory phase then continues for 24–48 h involving infiltration of neutrophils, followed by macrophages and lymphocytes. The proliferation phase, however, involved re-epithelialization, angiogenesis, collagen synthesis and extracellular matrix formation. This phase will take place around 72 h after wound occurrence. The wound healing process ends with the remodeling phase which can last for months and years. During this phase, the extracellular matrix undergoes remodeling to resemble the structure of normal skin tissue. Reorganization and cross-linking will continue its process from months to years depending on the seriousness of the wounds [3,4]. Despite the multiple biochemical constituents' function as building blocks in different healing phases, the wound healing process is dependent on immune mediators such as growth factors, cytokines, and chemokines by signaling to target cells and subsequently stimulating activities such as fibroblast proliferation, collagen synthesis, angiogenesis and others [5].

Natural plants have the potential to be used as wound healing agents in the future. The utilization of plants in wound healing is important to improve healing processes and reduce the financial burden of the patients [6]. The Zingiberaceae family is famously known for its medicinal potential, especially in Southeast Asia. This family has been used traditionally as medication to treat various symptoms and conditions including cough, vomiting, diarrhea and stomachache. There are at least 160 species from 18 genera of this family found in Peninsular Malaysia [7,8]. *Zingiber zerumbet* (L.) Smith, or locally known as Lempoyang in the Malay community, is one of the species from the genus *Zingiber* of the Zingiberaceae family which can be found in most tropical countries including Malaysia [8,9]. The *Z. zerumbet* rhizome has long been used as a traditional medicine to treat edema, loss of appetite, diabetes, inflammation and bronchitis [10]. It has properties that can aid wound healing such as antioxidant, anti-inflammatory, anti-bacteria and anti-fungus [10–12]. In addition, *Z. zerumbet* was proven to accelerate wound healing in preliminary study on Wistar rats [13]. Based on our preliminary data, an in-depth study is conducted to assess the wound healing potential of this plant.

In this experiment, full skin thickness dermal wounds were induced on rats and treated with 10 % ethyl acetate rhizome extract of *Z. zerumbet* (10 % ZZRE). Its effects on the percentage of wound closure and biochemical wound markers (total protein, hexosamine, uronic acid and hydroxyproline levels) were determined, and histological changes were observed. Despite the ability of *Z. zerumbet* in accelerating wound healing process [12], the influence of its reaction on immune mediators during various phases of wound healing had not been studied before. Thus, this study further investigates the effects of 10 % ZZRE on the level of immune mediators during wound healing process, which are the transforming growth factor-β1 (TGF-β1) and interleukin-10 (IL-10). TGF-β1 plays an important role especially in controlling cell growth, proliferation, differentiation, motility and apoptosis. As for IL-10, it is an anti-inflammatory cytokine that has been shown to have antifibrotic properties and plays an important role in wound healing processes [14].

2. Materials and methods

2.1. Experimental protocol

This study involved 90 adult male rats (*Rattus norvegicus*) of the Wistar strain, weighing between 220 and 320 g. These rats were obtained from the Animal Resource Unit Laboratory, Faculty of Medicine, Universiti Kebangsaan Malaysia, and were fed with rat pellets (Altronim Standard Diet) and water ad libitum. The use of these animals was approved by the Animal Ethics Committee of Universiti Kebangsaan Malaysia (UKMAEC) with approval number FSK/BIOMED/2012/ASMAH/21-NOV./473-NOV.-2012-APR-2013 (Appendix A and B).

The rats were then divided into three treatment groups: 10 % *Zingiber zerumbet* crude extract, positive control (solcoseryl gel), and negative control (PBS). The Solcoseryl gel was chosen as a positive control because this gel is widely used to promote wound healing. It is especially utilized for treating venous stasis ulcers, pressure sores, acute and chronic skin inflammation (dermatitis), and secondary and tertiary burns. Solcoseryl gel also aids in the refunctionalization of tissue that has been reversibly damaged due to hypoxia or lack of nutrients, accelerating and enhancing the quality of the healing process for lesions. As for PBS, it was chosen as a negative control because it is an isotonic and non-toxic solution for cells. PBS is also able to prevent cells rupturing or shrivelling up due to osmosis.

Each treatment group was further divided into five subgroups based on the day the rats will be sacrificed, with each subgroup consisting of six rats. The rats were anesthetized through the intravenous administration of ketamine, zoletil and xylazine cocktail (0.1 ml/100 g body weight). The dorsal part of each rat was shaved and disinfected with 70 % alcohol. Then, 6 mm biopsy punches were used to create six full skin-thickness wounds on the rat's dorsal body bilaterally.

Each rat that had been induced with wounds was placed in different cages to prevent the wound from getting worse, as rats are prone to biting each other. The bedding for rats that have been induced with wounds are changed every day. The cage was cleaned to reduce the possibility of bacterial or fungal contamination that can cause infection in rats' wounds. Physical observations were made on the rats throughout the study period starting from day one (24 h) after wound induction until the fourteenth day. The weights of the rats were also recorded before the wound induction and before the rats were killed to ensure they were in good condition and healthy. Treatment was applied topically to the wounds of rats daily until they were sacrificed. Six rats from each treatment group were sacrificed on the 1st, 3rd, 6th, 10th and 14th day.

2.2. Plant materials

The *Z. zerumbet* rhizomes used in this study were collected from a herbal farm in Temerloh, Pahang, Malaysia. The plant sample was sent to the university's herbarium and given a voucher specimen number UKMB 29952.

2.3. 10 % ethyl acetate extract of Z. zerumbet rhizomes (ZZRE)

The fresh rhizomes of *Z. zerumbet* were cleaned to remove any soil and chopped into thin slices and air-dried at room temperature. The dried rhizomes were then grinded into powder form and dried again at 40 °C in an oven to leave it dried completely. The powder was then soaked in hexane for 3 days. The resultant extracts were pooled and filtered to get the hexane extract of *Z. zerumbet* rhizomes. The process of soaking and filtering was repeated three times. Then, the same process was repeated using ethyl acetate to get the ethyl acetate extract of *Z. zerumbet* rhizomes. A rotary evaporator was then used to concentrate the ethyl acetate extract of *Z. zerumbet* rhizomes. The concentrated semi solid extract was put under the fume chamber and evaporated to dry. The presence of phytochemical constituents of *Z. zerumbet* ethyl acetate extract was detected using gas chromatographic analysis (GC-MS) and has been subsequently featured in our previous publication [15].

Prior to use, the extract was dissolved in dimethyl sulfoxide (DMSO) at a 1:1 ratio that is 100 g extract to 100 ml DMSO and diluted in phosphate buffered saline (PBS) with the proportion of 1:9 that is 10 ml extract to 90 ml PBS to produce 10 % ethyl acetate extract of *Z. zerumbet* rhizomes.

2.4. Macroscopic examination

Photographs of the wounds were taken on the day of wounding and the day they were to be sacrificed (Day-1, Day-3, Day-6, Day-10 and Day-14) to compare the gross or macroscopic appearance of the wounds between different treatment groups.

2.5. Determination of wound closure percentage

The wounds size on the day of wounding and the day of sacrificing were traced on transparent plastic and the surface area of the wounds was measured using graph paper. The wound contraction percentage was calculated by this formula: [1 - (wound surface area on the day of sacrificing/wound surface area on the day of wounding)] $\times$ 100.

2.6. Biochemical analysis

The wound area was cut with a sharp surgery knife and stored at −40 °C. The biochemical tests involved in this study included total protein, hexosamine, uronic acid and hydroxyproline levels.

2.6.1. Total protein concentration

Tissue homogenate were prepared using a tissue homogenizer at low temperature (on ice) to determine the total protein of wound tissue, following method described by Bradford (1976) [16]. The homogenates were then centrifuged for 3 min at 3000 rpm at 4 °C. The supernatants were stored in a freezer at −40 °C until further tested. To prepare 100-fold dilution, 0.1 ml of the tissue supernatant as mixed with 9.9 ml of phosphate buffer (PBS). Next, 0.1 ml of the diluted supernatant was combined with 5.0 ml of Bradford reagent and mixed thoroughly. The optical density of the total protein concentration of the tissue homogenates was read at 595 nm using a spectrophotometer.

2.6.2. Uronic acid

The procedure for preparing tissue hydrolysate and assessing the uronic acid content in wound tissue, followed the method outlined Bitter and Muir [17]. Initially, the wound tissues were thawed, cleaned, and then dried in the oven at 60 °C for 72 h. For digestion, 0.1 g of wound tissues was combined with 1 mg of papain in 10.0 ml of 0.5 M acetate buffer (pH 5), which contained 0.005 M cystein and 0.005 M disodium EDTA, and incubated at 65 °C for 24 h. Afterward, 5.0 ml of filtered sulphuric acid was placed in a test tube and cooled to 4 °C, before slowly adding 1.0 ml of tissue hydrolysate and mixing thoroughly. The test tubes were covered with marbles and gently shaken on ice. The mixtures were then heated for 10 min at boiling temperature and subsequently cooled to room temperature. Next, 0.2 ml of carbazol was added and the mixture was shaken again, heated in a boiling water bath for 15 min, and allowed to cool to room temperature. The optical density was measured at 530 nm.

2.6.3. Hexosamine

The tissue homolysate for hexosamine estimation was prepared following Cheng [18], with some modifications. Twenty milligrams of wound tissue were mixed with 2.5 ml of 4.0 N of hydrochloric acid. The test tubes were covered with marbles and incubated at boiling temperature for 4 h. Afterward, distilled water was added to brong the total volume to 10.0 ml. for each 2.0 ml of filtered hydrolysate, 1.0 N of sodium hydroxide was added to neutralize the pH. The hexosamine level was assessed using the method outlined by Isabel [19]. One milliliter of tissue hydrolysate was combined with 1.0 ml of freshly prepared acetylacetone reagent. The test tubes were again covered with marbles and incubated at boiling temperature for 15 min, then allowed to cooled to room temperature. Next, 5.0 ml of 95 % ethanol was added and mixed, followed by the addition of 1.0 ml of Ehrlich reagent, which was also mixed together

Finally, a 95 % ethanol was added to reach a final volume of 10.0 ml. The optical density was then measured at 530 nm.

2.6.4. Hydroxyproline

The hydroxyroline level in the wound tissue was measured using the method described by Stegemann and Stalder [20]. First, the wound tissues were cleaned and then dried in an oven at 60–70 °C for 12–18 h. For each 0.1 g of wound tissues, 1.0 ml of 6M hydrochloric acid was added, and the mixture was then homogenized using tissue homogenizer. The resulting tissue homogenate was

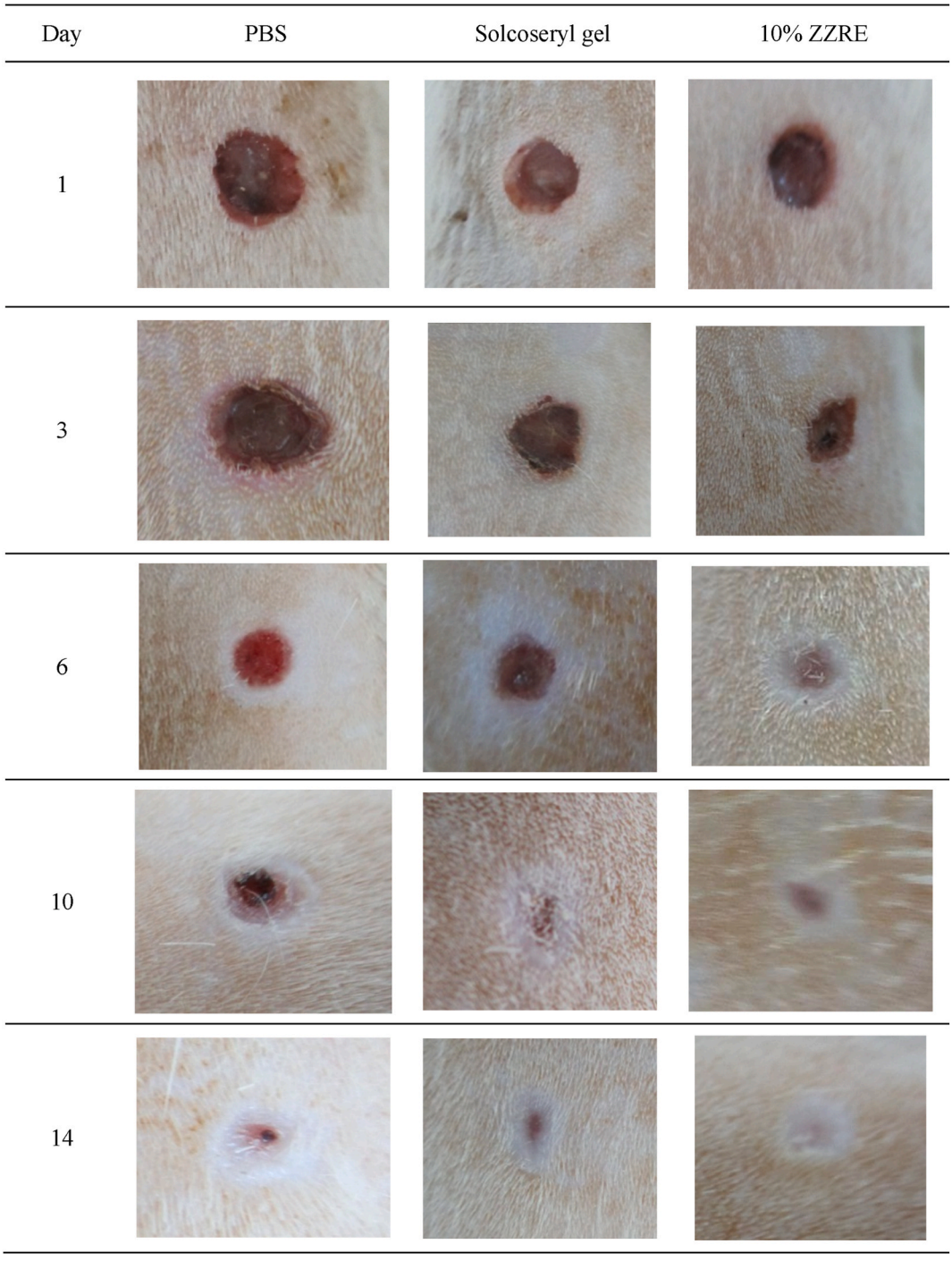


Fig. 1. Photographs of wounds treated with PBS, Solcoseryl gel and 10 % ZZRE on different days. Wounds treated with 10 % ZZRE showed faster and better healing compared to PBS and Solcoseryl gel group.

hydrolyzed in a boiling water bath for 4 h. Afterward, 1.0 ml of the supernatant was placed in a test tube and dried with a stream of nitrogen gas. The dried homogenate was then reconstituted with 2.0 ml of buffer solution. From this, 0.1 ml of mixture was further mixed with 1.0 ml of buffer solution. Next, 1.0 ml of chloramine T was added, mixed, and left at room temperature. After that, 1.0 ml of freshly prepared perchloric acid-aldehyde reagent was added and gently shaken until a pink color developed without any visible lines (schlieren). The optical density was then read at 550 nm.

2.6.5. Measurement of immune markers of the wounds (total TGF-β1)

The total TGF-β1 concentration in homogenate supernatants was determined using an ELISA kit obtained from California, according to the protocol of the manufacturer. In brief, standards and diluted samples were pipetted into each well of anti-TGF-β1 precoated microplate. Then, the microplate was washed, and the TGF-β1 detection antibody solution was added to each well. Following the washing, the Avidin-Horseradish Peroxidase D solution was added to each well. A substrate solution was then added to each well after washing, resulting in a blue. The colour development was halted by adding a stop solution. Absorbance was measured at a wavelength of 450 nm using a microplate reader. A standard curve was plotted, and the concentration of total TGF-β1 in the samples was determined by referencing this curve. The concentration obtained from the standard curve was the multiplied by the dilution factor to calculate the actual concentration of TGF-β1 in the samples.

2.6.6. Measurement of immune markers of the wounds (interleukin-10, IL-10)

The IL-10 concentration in homogenate supernatant was determined using an ELISA kit for total IL-10 obtained from Biorbyt, United Kingdom, according to the protocol of the manufacturer. In brief, standards and samples were pipetted into each well of an anti-IL-10- precoated microplate. Then, the microplate content was discarded and biotinylated antibody solution was added to each well. Following a washing step, an avidin biotin complex solution was added to each well. Substrate solution was then added to each well after the washing and a blue colour was then seen. The colour development was stopped by adding stop solution and the reading was taken at 450 nm using a microplate reader. A standard curve was plotted, and the concentration of the samples was determined by referring to the standard curve.

2.7. Statistical analysis

Statistical analysis of the results was performed using SPSS (Statistical Package for the Social Sciences) version 21.0 for Windows software. The data are presented as mean ± standard error of the mean (S.E.M). One-way ANOVA was conducted, followed by a Post-hoc Tukey test, with $p < 0.05$ deemed significant between groups.

2.8. Histological analysis

One of the six wounds was used as the specimen for histological analysis based on hematoxylin and eosin (H&E) staining. Each of the wound tissues was cut into half using forceps and surgical scissors. Histological slides were prepared by fixation in 10 % formalin solution, dehydration, clearing, infiltration, tissue embedding, dewaxing and staining.

2.9. Transmission electron microscopy observation

One of the six wounds was used as the specimen and fixed in 3 % glutaraldehyde for Transmission Electron Microscopy (TEM) preparation. The samples were processed and viewed at the Electron Microscopy Department of Universiti Kebangsaan Malaysia Medical Center (UKMMC).

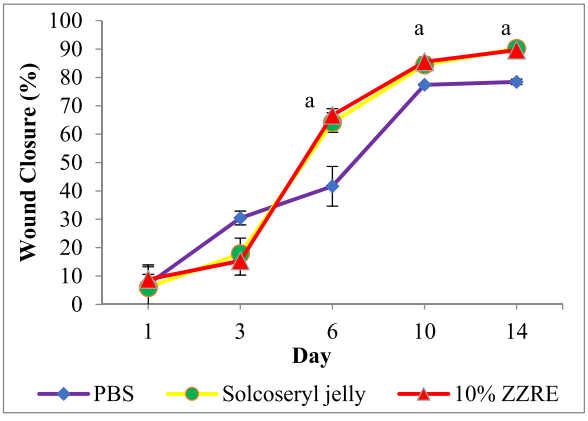


Fig. 2. Wound contraction percentage between treatment groups on different days. The data are expressed as mean ± SEM.
[a] $p < 0.05$ between 10 % ZZRE and PBS group.

3. Result

3.1. Macroscopic observation

Macroscopic observations (Fig. 1) show that the 10 % ZZRE group and Solcoseryl gel group healed with an almost similar rate. The wounds treated with 10 % ZZRE showed progressive wound contraction starting on Day-3. The scab of the wound detached earlier in the Solcoseryl gel and 10 % ZZRE groups as compared to the PBS group. From the photographs, the wounds of 10 % ZZRE seem to heal and dried faster than the Solcoseryl gel and PBS. On Day-10 and Day-14, the wound of 10 % ZZRE displayed smaller wound size and healed almost completely, whereas the wound of the PBS group is still wet, and the scab is still intact.

3.2. Wound closure percentage

The wound closure percentage was calculated (Fig. 2), and the result showed that the 10 % ZZRE treated wounds percentage was significantly ($p < 0.05$) higher than the PBS treated wounds on Day-6, Day-10 and Day-14. This strongly suggests the good healing effects of the ZZRE treated wound when compared to the untreated (PBS group).

3.3. Total protein

As shown in Fig. 3, the total protein concentration of the wound tissue treated by 10 % ZZRE was significantly lower than the PBS group at Day-1, but significantly ($p < 0.05$) higher at Day-14, compared to thePBS and Solcoseryl gel.

3.4. Hexosamine and uronic acid

Hexosamine was found to be in high concentration in all three groups at the initial of wound injury (Fig. 4). However, a decreasing pattern was observed in 10 % ZZRE group and there was significant ($p < 0.05$) low level at Day-10 and Day-14, comparing to both PBS and Solcoseryl gel groups. Meanwhile in Fig. 5, the uronic acid level of 10 % ZZRE group was detected significantly ($p < 0.05$) higher than the PBS group at Day-3 and Day-6. At Day-14, the 10 % ZZRE shows higher level than the Solcoseryl gel group, significantly ($p < 0.05$).

3.5. Hydroxyproline

Hydroxyproline was found to be significantly ($p < 0.05$) low level in the 10 % ZZRE group compared to both PBS and Solcoseryl gel groups at Day-1 (Fig. 6). The level of this parameter subsequently increased significantly ($p < 0.05$) as highest level in the 10 % ZZRE treated wounds as early as Day-3 till Day-14.

3.6. TGF-β1

The level of total TGF-β1 was highest on the wound tissue of the 10 % ZZRE group on Day-1 to Day-14 compared to other groups and significantly ($p < 0.05$) higher than the PBS group on day-14 (Fig. 7).

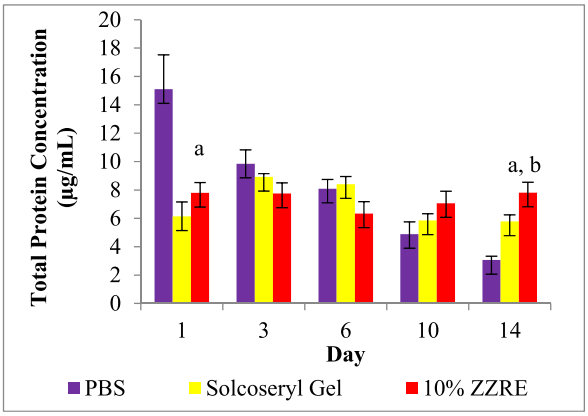


Fig. 3. Total protein concentration between treatment groups on different days. The data are expressed as mean ± SEM.
[a] $p < 0.05$ between 10 % ZZRE and PBS group.
[b] $p < 0.05$ between 10 % ZZRE and Solcoseryl gel group.

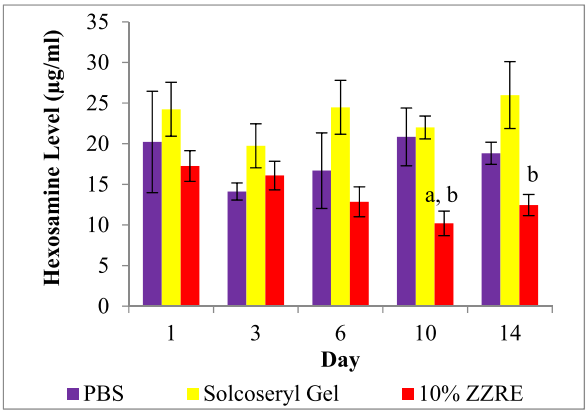


Fig. 4. Hexosamine levels between treatment groups on different days. The data are expressed as mean ± SEM.
[a] $p < 0.05$ between 10 % ZZRE and PBS group.
[b] $p < 0.05$ between 10 % ZZRE and Solcoseryl gel group.

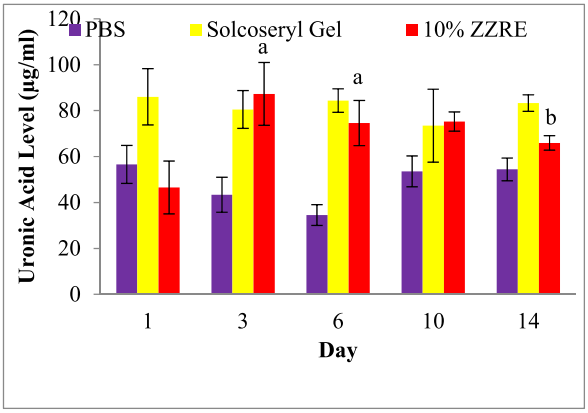


Fig. 5. Uronic acid levels between treatment groups on different days. The data are expressed as mean ± SEM.
[a] $p < 0.05$ between 10 % ZZRE and PBS group.
[b] $p < 0.05$ between 10 % ZZRE and Solcoseryl gel group.

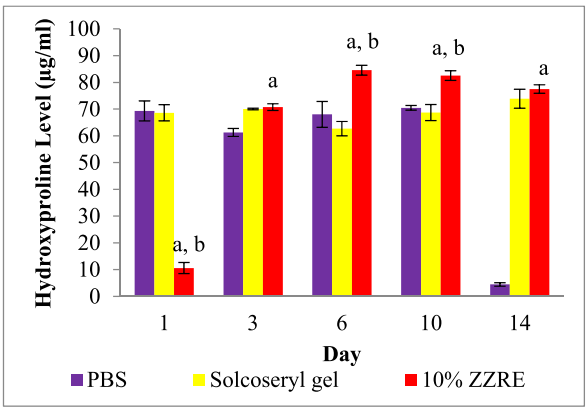


Fig. 6. Hydroxyproline levels between treatment groups on different days. The data are expressed as mean ± SEM.
[a] $p < 0.05$ between 10 % ZZRE and PBS group.
[b] $p < 0.05$ between 10 % ZZRE and Solcoseryl gel group.

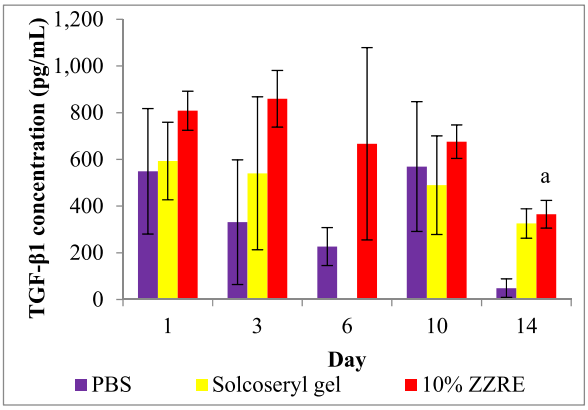


Fig. 7. TGF-β1 concentration between treatment groups on different days. The data are expressed as mean ± SEM.
[a] $p < 0.05$ between 10 % ZZRE and PBS group.

3.7. IL-10

IL-10 showed to be significantly lower at Day-1 in the 10 % ZZRE group. It slowly increased and reached its highest peak at Day-10, significantly ($p < 0.05$). The level of IL-10 is significantly ($p < 0.05$) higher at Day-14, compared to the PBS group (Fig. 8).

3.8. Histological observation

The histological analysis in Fig. 9 showed the healing progress for all groups. On Day-1, the infiltration of inflammatory cells, especially neutrophils, can be seen on all treatment groups. The scabs were observed in all groups until Day-3, but slowly detached in the 10 % ZZRE and Solcoseryl gel group at Day-6. A proliferation phase observed at Day-6 in all groups characterized by the presence of granulation tissue. On Day-10 and Day-14, wounds treated with 10 % ZZRE completely healed with full formation of epidermis. Hair follicles and sebaceous glands have formed back in this group as compared to the newly closed wounds in PBS group.

3.9. Transmission electron microscopic (TEM) observation

The presence of collagen was viewed under TEM as strip pattern of the collagen bundle at Day-1, Day-6 and Day-10 (Fig. 10). At Day-1, all groups showed the similar low content of collagen. The collagen started its deposition at Day-6 and was found abundant in the 10 % ZZRE group compared to both PBS and Solcoseryl gel groups at Day-10.

4. Discussion

Wound healing is a complex process of different phases that leads to the recovery of tissue integrity. Indeed, wound healing is prioritized in therapeutic research studies due to the challenges caused by wounds in various clinical complications. Several medicinal

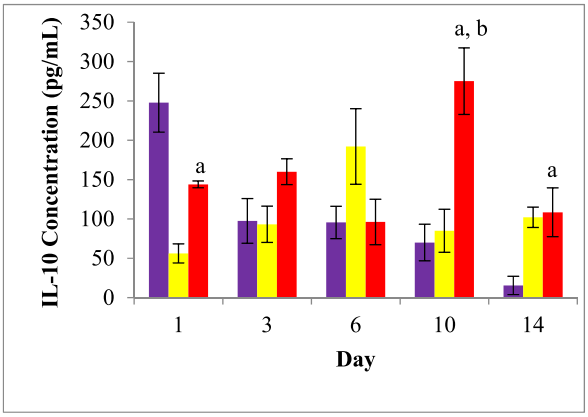


Fig. 8. IL-10 concentration in the wound tissues between treatment groups on different days. The data are expressed as mean ± SEM.
[a] $p < 0.05$ between 10 % ZZRE and PBS group.
[b] $p < 0.05$ between 10 % ZZRE and Solcoseryl gel group.

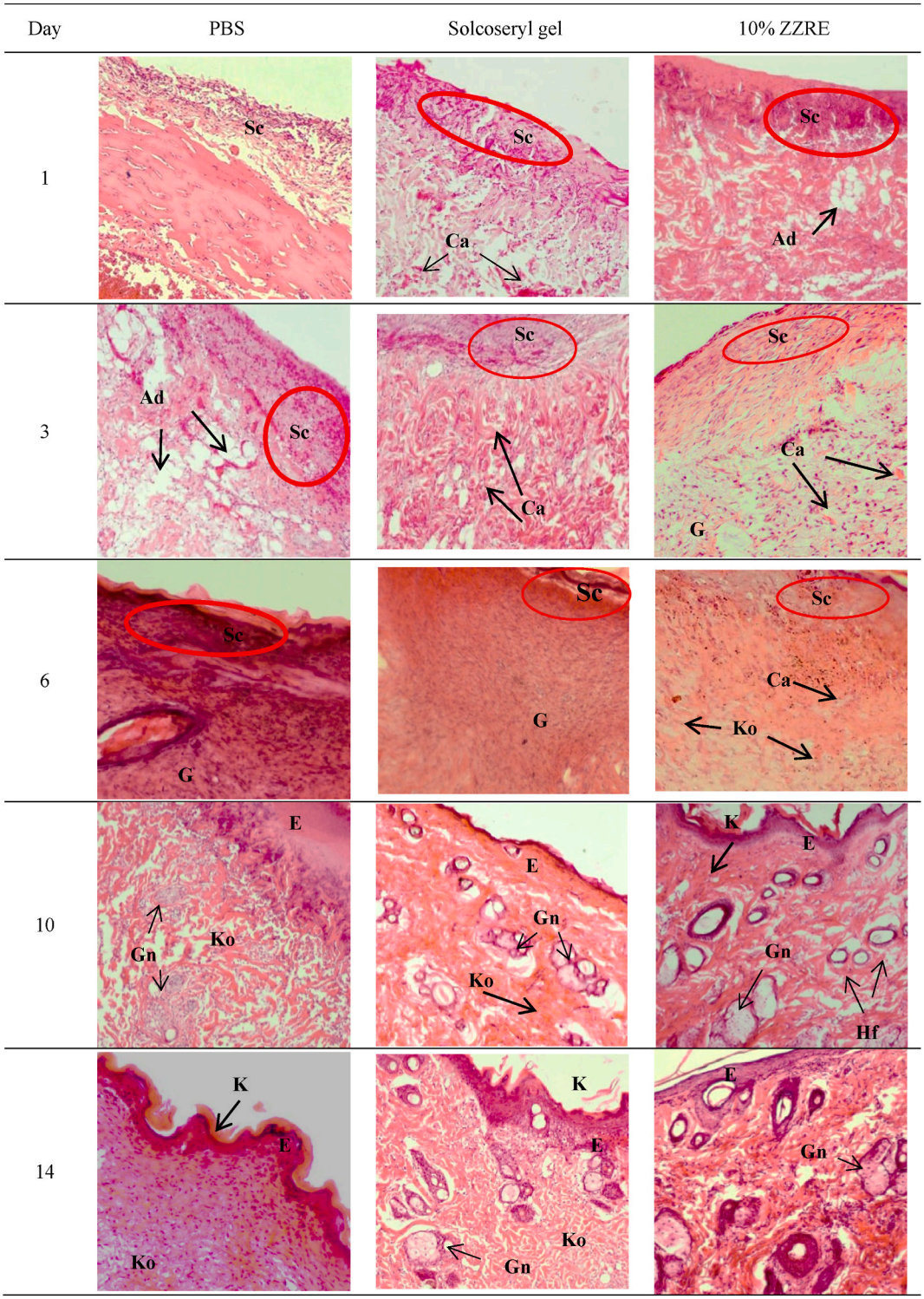


Fig. 9. The histopathological observation of wound healing from Day-1 to Day-14. Stained with H&E, at 100× magnification. The arrows pointing events during wound healing; Ad: adipose tissue, Ca: capillaries, E: epidermis layer, G: granulation tissues, Gn: sebaceous gland, Hf: hair follicle, K: keratin, Ko: collagen, Sc: scab.

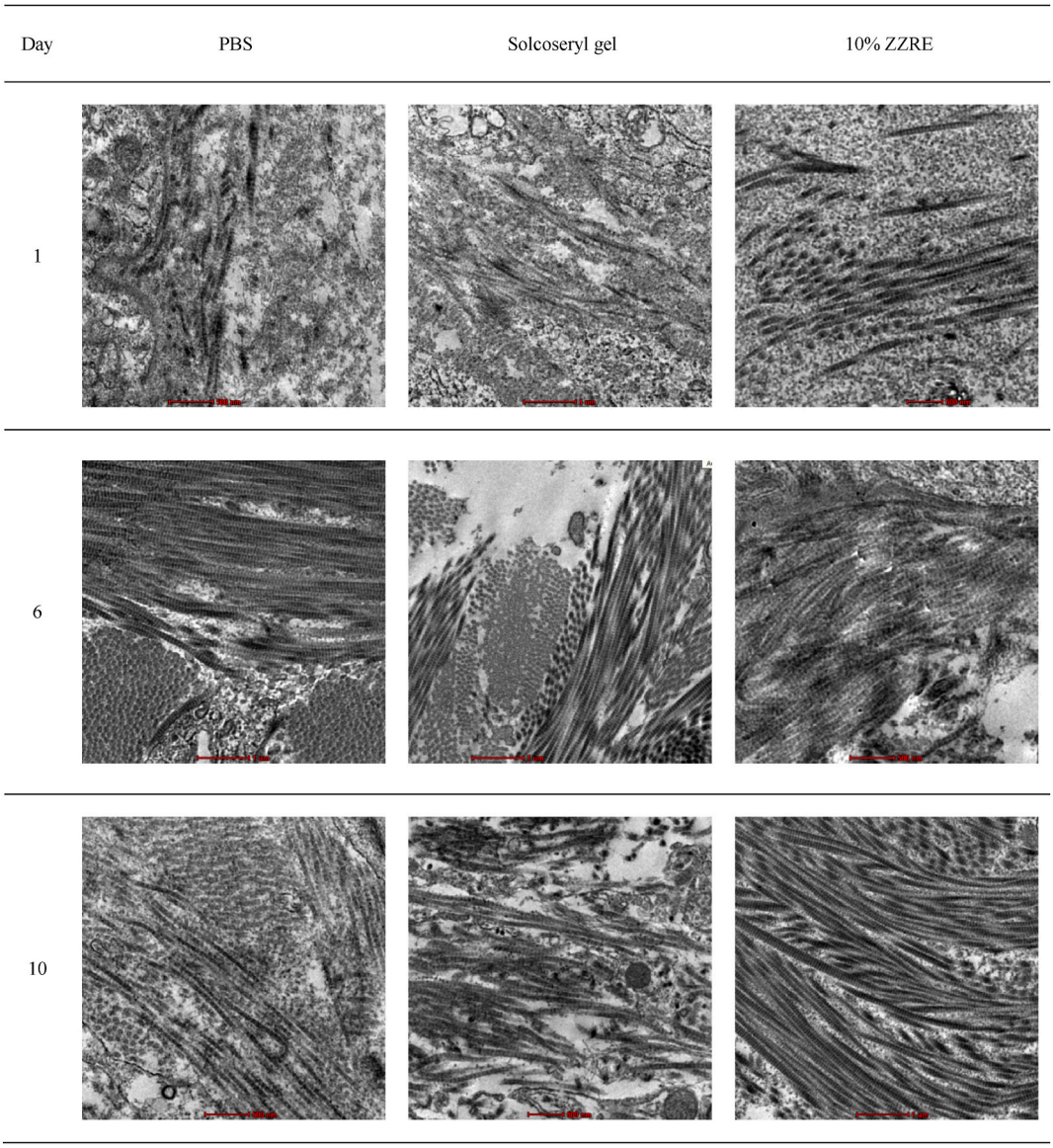


Fig. 10. Transmission electron microscopy view of collagen deposition at wound site on Day-1, Day-6 and Day-10 at 10,000× magnification.

plants have been discovered to be able to accelerate the process of wound healing. Various plant-based studies related to wound healing have also been reported and most of them have been proven to have positive effects in healing cascades [2,21–23].

In this study, the ethyl acetate extract of the *Z. zerumbet* plant was chosen as it has been reported previously that this extract contains the most quantity of *zerumbone* as reported by Sam et al. [15]. A recent study by Liu et al. [24] also suggests that zerumbone could be a valuable agent in enhancing cutaneous wound healing, as evidenced by accelerated wound closure, improved tissue regeneration, and favorable changes in inflammatory and growth factors. Besides that, the ethyl acetate extract has shown good hepatoprotective [25], nephroprotective [10], neuroprotective [26], anti-gouty arthritis [27] and many other properties.

The choice of 10 % ZZRE concentration to treat the wound is based on the commercial topical wound treatment in the market that used mostly 5–10 % of the concentration of the active ingredient. According to Young and McNaught [28], contraction of the wounds usually starts on Day-7 after injury has occurred. The contraction is largely regulated by myofibroblast as it pulls the edges of the wound together. Thus, this shows that the 10 % ZZRE is effective in wound contraction which involves the activation of fibroblast differentiation. This characteristic has been proven by the ZZRE extract on an in vitro model of wound healing as it showed a faster movement of human adult cell line in a Scratch Assay test [29].

Extravasation and blood clots formed after skin injury, trigger hemostasis. Neutrophils will be attracted towards the site of the wound due to chemical mediators secreted in abundance, including interleukin-1 and tumor necrosis factor-α, which eventually contribute to the inflammatory phase [30]. Barany [31] stated that the concentration levels of total protein will show a significant

increase in inflammation. Thus, the increase of protein levels at the initial wounds may indicate the presence of chemical mediators that are involved in the hemostasis and inflammatory phase. The low protein levels in the 10 % ZZRE group supported Chien et al. [32] study which showed that *Z. zerumbet* possessed bioactive anti-inflammatory compounds. The decreasing pattern seen in the protein levels portrayed the end of the inflammatory phase as days passed [33]. However, the increment in total protein levels at Day-10 and Day-14 is contributed by the deposition of collagen, the main extracellular protein in the granulation tissue of the wound. The increased level of total protein is due to the synthesis by fibroblast cells to provide strength and integrity for the tissue matrix [34].

Rapid synthesis of extracellular matrix is the main cellular event occurring in the proliferative phase, with glycosaminoglycans (GAG) and proteoglycans being the ground substances of the matrix. Hexosamine and uronic acid as the matrix molecules, combine to form hyaluronan, which is a type of GAG and plays an important role in tissue hydration [35]. Increasing levels of hexosamine and uronic acid prepare a sufficient fluid matrix scaffold that aids in early tissue remodeling [36–38]. The results are paralleled with the study by Rees et al. [35], where high hexosamine levels on Day-1 for all three groups suggested that the GAG is being synthesized as the skin substratum was occurring rapidly, and subsequently lowered indicating collagen deposition took over at the remodeling phase.

Hydroxyproline is the main component detected almost exclusively in collagen [34,39]. The formation of collagen occurs at a rapid rate at initial wound healing but is subsequently broken down by collagenase at the remodeling phase, for cross linking of collagen to occur resulting in an increase in wound tensile strength. The deposition of collagen will then continue to increase as the wounds heal [23]. The notable rise in collagen levels in the 10 % ZZRE group on Day 3 and Day 6 was attributed to the enhanced migration of fibroblasts and epithelial cells to the wound site. This aligns with our earlier findings from an in vitro wound healing model, which demonstrated that the ethyl acetate extract of Zingiber zerumbet could stimulate cell proliferation and increase the migration rate of the human dermal fibroblast adult cell line (HDF-a) [29]. Conversely, the reduction in collagen content especially in the PBS group is postulated that the inflammatory phase may have been prolonged, and this led to more collagen degradation than collagen synthesis [40].

TGF-β1 plays an important role in various stages of wound healing. It promotes the production of components of extracellular matrix such as collagen and fibronectin. It helps in the stabilization and maturation of the scar, in addition to the formation of granulation tissue by stimulating the expression of collagen I, collagen III and elastin [41]. Furthermore, it stabilizes the extracellular matrix for further collagen deposition. IL-10 plays a role in reducing the inflammatory response. As shown in the findings, IL-10 concentration at the wound of the 10 % ZZRE group was significantly lower than PBS group on Day-1, indicating the anti-inflammatory activity of *Z. zerumbet* extract. IL-10 decreases the leukocyte infiltration to the inflammation site and inhibits the synthesis of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) [42,43]. IL-10 also inhibits the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1β from leukocytes. On the other hand, it was shown to be higher than the PBS group on Day-10 and Day-14. IL-10 helps in the synthesis of hyaluronan which is one of the components of the extracellular matrix through its receptor IL-10R1 and activation of signal transducer and activator of transcription 3 (STAT3) [44]. According to Meran et al. [45], hyaluronan facilitates TGF-β1 to regulate proliferation of the fibroblast through Smad3 signaling. This suggested that 10 % ZZRE may help in the proliferation of fibroblasts and the formation of extracellular matrix through the action of IL-10.

From histological findings, the 10 % ZZRE group had a better progression in healing compared to the PBS group and at par with Solcoseryl gel. Significant reduction of inflammatory cells was seen in the 10 % ZZRE group during Day-3 indicating a good anti inflammatory effect of *Z. zerumbet* [12]. This result further supported that 10 % ZZRE is able to reduce inflammation at the wound site.

Angiogenesis plays an essential role as a source of oxygen and important nutrients in proliferation and also migration of fibroblasts and epithelial cells. Early formation of blood vessels as shown in the 10 % ZZRE and Solcoseryl gel groups on Day-3 and Day-6 indicated the ongoing proliferation phase in wound healing. According to Young and McNaught [28], important events occurred during the proliferation phase of the wound healing process, including angiogenesis, re-epithelialization and granulation tissue formation. This showed that wounds treated with 10 % ZZRE entered the proliferation phase earlier compared to other groups.

On Day-6 and Day-10, wounds of all groups were already at the proliferation phase of wound healing which was characterized by the presence of re-epithelialization and connective tissue filled with blood vessels, fibroblasts, macrophages and lymphocytes. Dense collagen deposition was seen on Day-10 and Day-14. Furthermore, the effectiveness of collagen deposition can be supported by the transmission electron microscopy view. Not much difference could be observed on Day-14 as the wound had reached the healing stage. However, scar, epithelium and keratin could be observed on three treatment groups.

5. Conclusion

In summary, the study demonstrates that the ethyl acetate extract of *Zingiber zerumbet* rhizome (ZZRE) at a 10 % concentration significantly enhances wound healing in Wistar rats compared to the control treatments. The treatment group showed faster wound closure, improved protein levels, increased hydroxyproline, and elevated immunological markers such as TGF-β1 and IL-10, which are crucial for wound healing. Histological analysis further supported these findings, showing reduced inflammation and increased collagen deposition in the ZZRE-treated wounds. These results indicate that 10 % ZZRE is an effective agent for accelerating wound healing and has the potential for development as a future wound healing treatment. The promising results from this study suggest that further research of clinical trials will be necessary to confirm the safety and effectiveness of ZZRE in humans. The development of various formulations, such as creams or gels of ZZRE could also be explored to enhance its applicability and ease of use in clinical settings. While the study provides valuable insights into the wound healing properties of ZZRE, it has certain limitations. The study did not investigate the long-term effects of ZZRE treatment or its potential side effects, which are crucial for determining its safety profile. Also, the study was conducted solely on Wistar rats, and the results may not directly translate to other species or subjects. Addressing

these limitations in future research will be essential for the comprehensive evaluation of ZZRE as a wound healing agent.

CRediT authorship contribution statement

Asmah Hamid: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Pek Lian Chong:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yun Ying Khor:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Poh Ying Kong:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Nur Rasyiqin Rasli:** Writing – review & editing. **Nor Malia Abd Warif:** Writing – review & editing, Validation, Supervision, Project administration. **Ahmad Rohi Ghazali:** Writing – review & editing, Validation, Supervision, Project administration. **Nurul Farhana Jufri:** Writing – review & editing.

Data availability statement

The data of this study are included in this article. The additional data that support the findings of this study are available from the authors upon reasonable request.

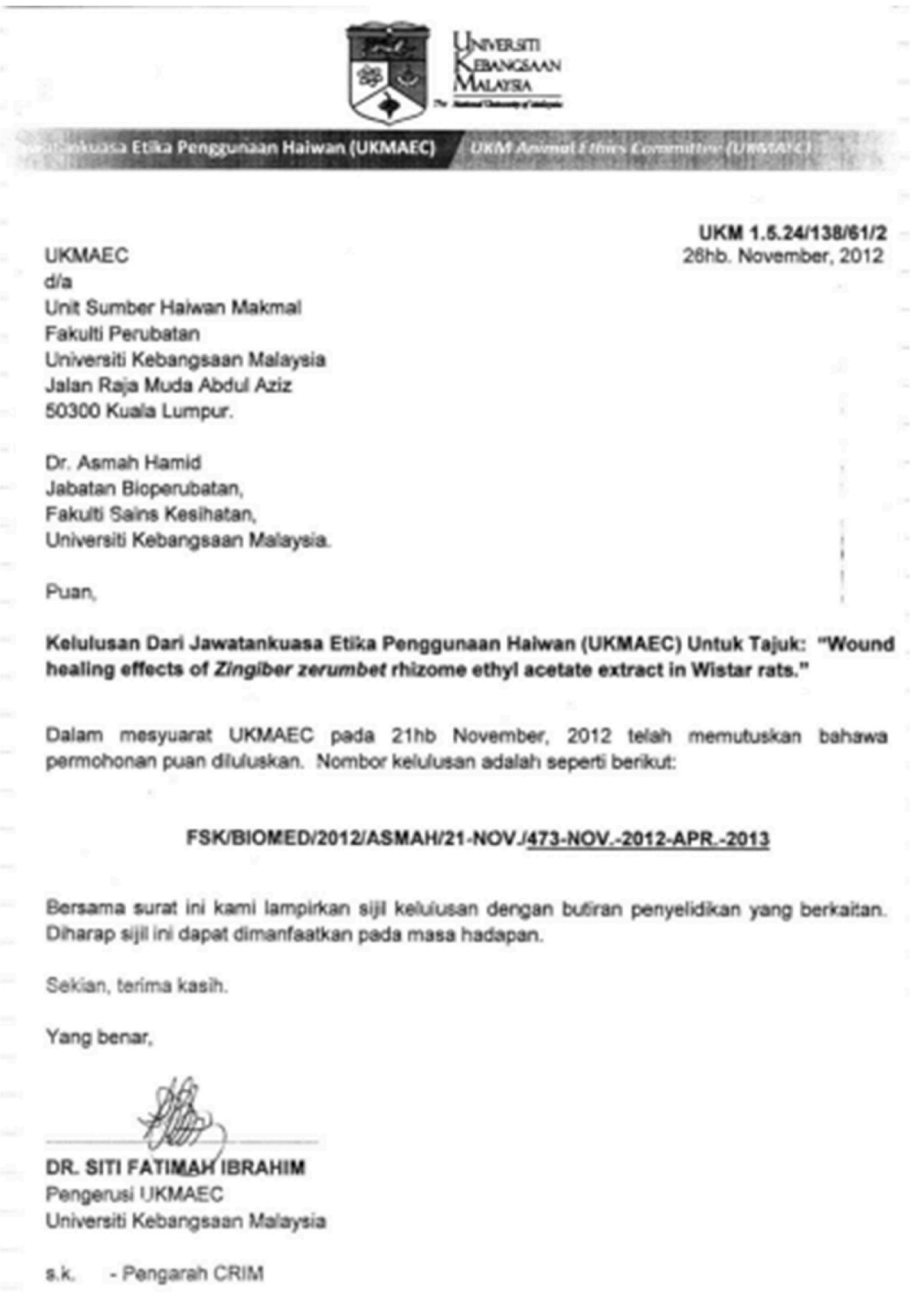
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Universiti Kebangsaan Malaysia for funding this project via the Research University Grant (Geran Universiti Penyelidikan), GUP-2016-076. The authors would also like to gratefully acknowledge the staffs of the Programme of Biomedical Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia for the help given throughout the research. We would like to thank all students, researchers and those who were directly or indirectly involved in this research.

Appendix A



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UKM 1.5.24/138/61/2
28hb. November, 2012

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Puan,

Kelulusan Dari Jawatankuasa Etika Penggunaan Haiwan (UKMAEC) Untuk Tajuk: "Wound healing effects of *Zingiber zerumbet* rhizome ethyl acetate extract in Wistar rats."

Dalam mesyuarat UKMAEC pada 21hb November, 2012 telah memutuskan bahawa permohonan puan diluluskan. Nombor kelulusan adalah seperti berikut:

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Bersama surat ini kami lampirkan sijil kelulusan dengan butiran penyelidikan yang berkaitan. Diharap sijil ini dapat dimanfaatkan pada masa hadapan.

Sekian, terima kasih.

Yang benar,

DR. SITI FATIMAH IBRAHIM
Pengerusi UKMAEC
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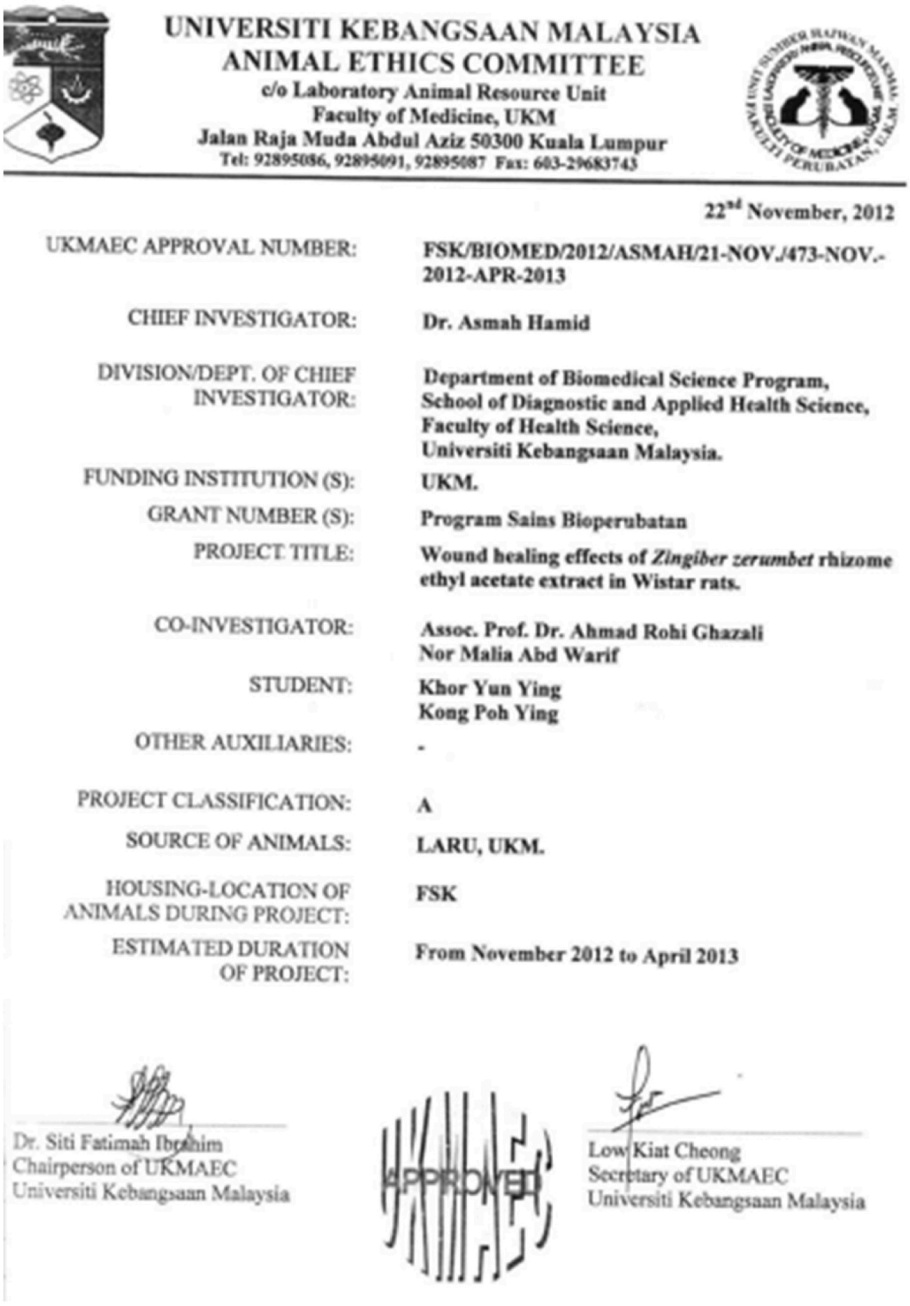
s.k. - Pengarah CRIM

Appendix B



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DIVISION/DEPT. OF CHIEF INVESTIGATOR:	Department of Biomedical Science Program, School of Diagnostic and Applied Health Science, Faculty of Health Science, Universiti Kebangsaan Malaysia.
FUNDING INSTITUTION (S):	UKM.
GRANT NUMBER (S):	Program Sains Bioperubatan
PROJECT TITLE:	Wound healing effects of *Zingiber zerumbet* rhizome ethyl acetate extract in Wistar rats.
CO-INVESTIGATOR:	Assoc. Prof. Dr. Ahmad Rohi Ghazali Nor Malia Abd Warif
STUDENT:	Khor Yun Ying Kong Poh Ying
OTHER AUXILIARIES:	-
PROJECT CLASSIFICATION:	A
SOURCE OF ANIMALS:	LARU, UKM.
HOUSING-LOCATION OF ANIMALS DURING PROJECT:	FSK
ESTIMATED DURATION OF PROJECT:	From November 2012 to April 2013

Dr. Siti Fatimah Ibrahim
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