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# *In vitro* and *in vivo* evaluation of the wound healing properties and safety assessment of two seaweeds (*Sargassum ilicifolium* and *Ulva lactuca*)

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ABSTRACT

Seaweeds have been regarded as a reservoir of biologically active molecules that are important in the pharmaceutical industry. The aim of the present study was to explore the wound healing properties and to assess the safety of the seaweed *Sargassum ilicifolium* and *Ulva lactuca.* Enhanced cell proliferation and cell migration activities were observed in L929 cells treated with *S. ilicifolium* extract compared to *U. lactuca* extract treated cells and the control group. In-vivo experiments were conducted using five groups (10 in each) of Albino mice (BALB/c). Mice in group I and group II were treated (Orally, 100 mg/kg BW/day) with aqueous extracts of *S. ilicifolium* and *U. lactuca*, respectively for 14 days. Treatment group III received a topical application of the aqueous extract of *S. ilicifolium* (25% w/w) and ointment base (75% w/w) (2 g/kg BW/day, for 14 days). Group IV (Control) received an equal amount of distilled water, orally and mice in group V kept without wounds. The extract from *S. ilicifolium* showed stronger wound healing properties than the one from *Ulva lactuca*. Histopathological findings also revealed that the healing process was significantly enhanced in the mice group treated orally with *S. ilicifolium* aqueous extract. These findings show that *S. ilicifolium* species possess promising wound healing properties *in-vitro* and *in-vivo*.

## 1. Background

A wound is an injury that occurs in a part of the body, especially one in which a break is made in the skin [1]. The wounds and their infections are challenging clinical problems frequently associated with morbidity and mortality in developing countries [2]. 77% of the deaths of surgical patients were confined to surgical wound infections [3]. In general, wound healing is a dynamic process that can be divided into five overlapping phases: hemostasis, inflammation, migration, proliferation, and maturation [4]. Since ancient times, humans have been using many plant resources based on empirical observations without any scientific knowledge for the treatment of wounds, cuts, and burns [5]. Therefore, drugs derived from medicinal plants are in great demand due to the common belief that they are safe, reliable, clinically effective, low cost, and better tolerated by patients [6]. Although there has been an enormous development in the pharmaceutical industry, wound healing drugs are still at an unsatisfactory level because of low availability, high cost, and with various detrimental side effects [7]. Marine algae have been used in traditional medicine, and the functional food industry for many centuries [8,9]. Therefore, many types of research are focused on seaweeds as a source with great potential for extracting new therapeutic compounds. Nevertheless, a wide range of anti-inflammatory, antibiotic, anti-HIV, anticoagulant, anticonvulsant [10], antineoplastic, antiulcer [11], and antimicrobial activities of seaweeds have also been reported [12]. Also, seaweeds are comprised of bioactive compounds that are capable of producing a great variety of secondary metabolites with

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broad biological activities [13,14]. The seaweeds can also be used to prevent tissue damage and stimulate the wound healing process [15]. Biologically active compounds present in a wide range of medicinal plants such as tannins, triterpenoids, and alkaloids have been found to affect one or more phases of the wound healing process [7,16]. Although many studies have been conducted on wound healing properties of medicinal plants, there is scant data on marine resources including seaweeds. Thus, the present study was conducted to explore the potential wound healing properties and safety of two abundant seaweed species *Sargassum ilicifolium* and *Ulva lactuca* collected from Sri Lankan coastal areas using *in vitro* and *in vivo* studies.

## 2. Methods

### 2.1. Seaweeds material and sample preparation

Seaweeds *Sargassum ilicifolium* J. Agardh 1848 (Ochrophyta: Phaeophyceae: Fucales: Sargassaceae) and *Ulva. lactuca* Linnaeus 1753 (Chlorophyta: Ulvophyceae: Ulvales: Ulvaceae) were collected in January 2015 from the south coastal algae beds, Ahangama (N05° 58.006′ E080° 22.482′) and Talpe (N05° 59.792′ E080° 16.898′) in Sri Lanka. The seaweeds were authenticated at the "National Herbarium of the Peradeniya Botanical Garden" and voucher specimens representing *S. ilicifolium* and *U. lactuca* were deposited in the Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya for future reference. Seaweeds were washed thoroughly first in seawater, then in tap water, and finally, in distilled water to remove sand particles, impurities, and epiphytes. Then the seaweed samples were dried, at 60 °C for four days until a constant weight was obtained. The samples were milled (final particle size ~ 0.5 mm) with an electrical grinder (Herbal Grinder CS-700, China) and stored at −20 °C.

### 2.2. Preparation of seaweed aqueous extract procedure

Approximately 100 g of seaweed powder was soaked in 500 ml of distilled water and it was kept for 1 h at 60 °C in an ultrasound sonicator (Branson 2510, Danbury, USA) to permit full extraction of the active ingredients. Then the samples were shaken in a roller (Denley-spiramix 5, UK) at room temperature. After three days the preparation was filtered using nylon mesh 0.50 μm and filtrates were collected. Finally, the extracts were kept at −20 °C in closed containers before use. The filtered extract was used for the *in vivo* experiments. The filtered extracts were centrifuged (Beckman Avanti, UK) at 15,000 rpm for 10 min at 4 °C and the supernatants were used for *in vitro* experiments.

### 2.3. In vitro experimentation

#### 2.3.1. Toxicity assay

The L929 cell line (mouse fibroblast) was purchased from American Type Culture Collection (ATCC), USA. Cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640), supplemented with 10% of Fetal Bovine Serum (FBS, Invitrogen, Gibco, UK), 10 ml/L Penicillin and Streptomycin (PSA), 2 g/L Sodium bicarbonate, and 5 ml/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cell cultures were maintained at 37 °C in a humidified 5% $CO_2$ incubator (MCO.20AIC, Japan). L929 cells were seeded in 96-well plates (Corning Glasswork, Corning, NY) at a concentration of $2 \times 10^5$ cells/well and incubated for 24 h to reach full confluence. The medium was removed from the wells which were washed with phosphate buffered saline (PBS) to remove dead cells and to obtain an evenly grown cell monolayer. RPMI medium separately supplemented with *S. ilicifolium* and *U. lactuca* extract was added 50 μl per well and incubated further for 24 h. Ten dilutions of a two-fold serial dilution were used with triplicates and diluent in eight wells were used for corresponding concentration. Negative and positive control tests were prepared using distilled water and absolute ethanol, respectively. 24 h later, the treatment solutions were removed from the wells which were washed again with PBS to remove any remaining traces. 10 μl of MTT (5 mg/ml in PBS) solution and 90 μl ascorbate free cell culture media, RPMI 1640 was added and further incubated for 4 h at 37 °C. The reaction was stopped by adding 100 μl DMSO (dimethyl sulfoxide, spectrophotometric grade), and finally, absorbance was measured using ELISA reader (Muitiskan Ex, German) at 570 nm wavelength. *In vitro* cytotoxic activity was measured by the MTT assay and all experiments were carried out in triplicate. Based on the results of the experiments, the extract which showed the highest proliferation activity was selected for further studies, and an extract with a neutral effect was selected as a control standard.

#### 2.3.2. Scratch wound healing assay

L929 cells (70–80% confluence, 24 h) were seeded into a 24-well tissue culture plate. Without changing the medium, cell culture monolayers were scratched with a sterile 200 μl pipette tip across the center of the well. While scratching across the surface of the well, the tip was kept perpendicular to the bottom of the well. After scratching, the wells were gently washed twice with medium to remove the detached cells. 300 μl of growth medium was added to each well and photographed using a camera attached to an inverted microscope to obtain the same field during the image acquisition; markings were created to be used as reference points close to the scratch. The cell culture medium was replaced immediately with 150 μl fresh medium supplemented with 150 μl *S. ilicifolium* (7.79 μg/μl) and *U. lactuca* aqueous extracts (47.72 μg/μl), to the corresponding wells containing the scratched cell monolayer. The wound gap was photographed (Nikon Coolpix 4500: Nikon, Tokyo, Japan) at 0 h, 12 h, and 24 h' time period to check the wound healing efficiency of the seaweeds extracts.

### 2.4. In vivo experimentation

#### 2.4.1. Dose selection for treatment

A preliminary dose-response study was conducted to determine the toxic effects on mice. Graded doses of aqueous extracts of seaweeds 100 mg/kg, 200 mg/kg, and 400 mg/kg were orally administered to mice once daily for 14 days. The amount of the seaweed extract dosage given to each animal was initially based on the calculated animal's body weight [17]. Animals were observed individually after the oral treatment at least once during the first 45 min, periodically during the first 24 and 48 h for behavioral changes and stimulation were monitored for 14 days. If mortality or weakness was observed the dose administered was assigned as a toxic dose. The result of the dose response study in toxicity indicated that all dosages of seaweed extracts were not toxic to the mice. Therefore, the minimum dose of 100 mg/kg of seaweed extract was chosen for further studies on assessment of various physical, biochemical, and histopathological parameters of wound healing on mice in excision wound models.

#### 2.4.2. Preparation of ointment

An ointment was formulated by mixing the aqueous extract of *S. ilicifolium* (25% w/w) with 75% w/w of commercially available excelsior (wool fat: 2.5 g, hard paraffin: 2.5 g, cetostearyl alcohol: 2.5 g, white soft paraffin: 42.5 g).

#### 2.4.3. Experimental animals and wound induction

Fifty, eight-week-old (body weight 28.73 ± 0.32 g) healthy albino BALB/c female mice were selected. They were kept in individual cages in a ventilated room with temperature regulated at 28 ± 2 °C, humidity 65 ± 5%, and with a 12 h light/dark cycle. They were fed with a standard pellet diet and tap water *ad-libitum*. All the mice were acclimatized for 7 days before the study and starved 12 h before administration of seaweed extracts or distilled water. All the surgical interventions were carried out under sterile conditions under general anesthesia. A full-thickness excision wound (64.73 ± 0.89 $mm^2$ in a circular area) was

induced on the shaved dorsolateral skin with an 8 mm Piercing sterile biopsy punch. The area was washed with normal saline (0.9% NaCl) immediately after wounding. The mice were then divided into five different groups: Treatment group I received *S. ilicifolium* extracts (100 mg/kg BW/day for 14 days, orally); Treatment group II received *U. lactuca* extracts (100 mg/kg BW/day for 14 days, orally); Treatment group III received the topical application of the aqueous extract of *S. ilicifolium* (25% w/w) and ointment base (75% w/w) (2 g/kg BW/day, for 14 days). Group IV (Control) received an equal amount of distilled water, orally and mice in group V kept without wounds. Changes in wound sizes were measured and reduction (wound contraction) in the wound area was expressed as a percentage of the original wound size [18]. Furthermore, the time taken to completely heal the wound was calculated [19].

Calculations were done as follows:

$$\text{Percentage of wound healing} = (\text{Total wound area} - \text{Present wound area})/(\text{Total wound area}) \times 100$$

Ethical approval for conducting the experiments with mice was obtained from the Ethical Review Committee of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka (Ref No. ERC/FVMA/UOP/2013/10). All experimental procedures and animal care had been approved by the Faculty Ethics Committee, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka.

#### 2.4.4. Toxicity study

Body weights of mice were recorded during the experiment period. Three mice from each group were sacrificed on days 3, 7, 10, 14, 21, and 28, after the initial oral administration (IOA). Blood was collected and serum was separated by centrifugation at 2500 rpm at 4 °C for 10 min. The clean serum was separated and used for the estimation of the serum enzyme levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine levels [20] using commercially available human diagnostic test kits (Human Wiesbaden, Germany) according to the manufacturer's instructions. The Erba Mannheim (Germany) spectrophotometer was used for the serum enzyme measurement.

#### 2.4.5. Histopathology

Vital organs (liver, kidney, heart, spleen, and lungs) of sacrificed mice were dissected, weighed, collected and a specimen sample of tissue from the healed wound was isolated from each experimental group of mice and fixed in 10% neutral buffered formalin (NBF), immediately. Formalin-fixed tissues were embedded in paraffin and sectioned at 3–5 μm in thickness. Formalin-fixed, de-waxed sections were stained with hematoxylin and eosin (HE) for histopathological observations.

#### 2.4.6. Estimation of haematological and serological parameters

Blood was collected from the experimental animals on days 0, 3, 7, 10, 14, 21, and 28, and analyzed for the following parameters using standard laboratory techniques. Total count number of WBC (White Blood Cells) differential were counted by examining a blood smear. The packed cell volume was determined by microhaematocrit centrifuge (England) at 12,000 rpm for 5 min and then read using microhaematocrit reader (Hawksley, England). Blood glucose levels were evaluated by using a commercially available human glucose liquicolor kit using Erba Mannheim (Germany) spectrophotometer. Total serum protein levels of experimental animals were evaluated on above mentioned days using an RHC-200 ATC refractometer (Germany).

### 2.5. Statistical analysis

Graph Pad Prism Version 4.03 for Windows (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. All the data obtained during the experimental period were statistically analyzed using one-way and two-way analysis of variance. Multiple comparisons between the significant levels of interactions of the variables were done by Turkey's method. Values were expressed as the mean ± SE and $p < 0.05$ was considered significant.

## 3. Results

### 3.1. In vitro experimentation

The impact of different concentrations of *S. ilicifolium* (0.49–7.79 μg/μl) and *U. lactuca* (2.98–47.72 μg/μl) extract on L929 cells were determined over the course of 24 h. The concentrations up to 7.79 μg/μl of *S. ilicifolium* extract and 47.72 μg/μl of *U. lactuca* did not show significant cytotoxic effect against L929 cells. Instead, it showed a cell proliferation activity in a dose-dependent manner. the cell proliferation activity was gradually decreased in concentrations lower than 0.49 μg/μl (Fig. 1).

The aqueous extract of *S. ilicifolium* has induced proliferation and/or migration of the mice fibroblasts (L929) with compared to *U. lactuca* treated cells and cells in the control group. 7.79 μg/μl was the best *S. ilicifolium* concentration which showed the highest cell proliferation and/or migration activity compared to all other concentrations of *S. ilicifolium* used for the experiment.

### 3.2. In vivo experimentation

During the experimental period, there were no noticeable behavioral changes in all experimental and control groups of mice. Moreover, food and water intakes of mice of all treatment and control groups were not changed significantly during the experimental period. These observations of short-term behavioral or dietary changes suggested that oral administration of *S. ilicifolium* and *U. lactuca* at the concentrations tested in this study have not caused any significant changes. Seaweed ointment formulations did not produce any skin irritation for about a week when applied over the skin.

#### 3.2.1. Wound healing study

When compared, the effectiveness of the oral administration and the topical application of the *S. ilicifolium,* the oral application had better wound healing activity. *U. lactuca* had no significant wound healing effect compared to the control group. The rate of wound contraction was expressed ($mm^2$) as the reduced wound area (Table 1).

Mice treated with extract of *S. ilicifolium* exhibited significantly enhanced level of wound healing activity within three days ($p < 0.05$) when compared with *U. lactuca* treatment group and control group. In the control group, the measurement of wound open area on the third day of observation was $42.21 \pm 3.20$ $mm^2$ whereas wound open area of mice groups orally treated with aqueous extracts of *S. ilicifolium* and *U. lactuca* (100 mg/kg) was recorded as $32.73 \pm 2.83$ $mm^2$ and $44.68 \pm 3.77$ $mm^2$ respectively. The open wound area of the *S. ilicifolium* tropical treated mice was $39.00 \pm 5.92$ $mm^2$. The progressive healing changes occurred in wounds of mice of each group during the experiment are given below (Fig. 2).

The statistical analysis showed that at the end of the study period the oral treatment with an aqueous extract of *S. ilicifolium* (100 mg/kg) caused a significant contraction of the wound compared to the control group.

*3.2.1.1. Histopathological studies of wounded tissues.* Images were taken under microscope × 400 (Carl Zeiss Microscopy, Germany). Histopathological findings showed that the oral administration of aqueous extract of *S. ilicifolium* has enhanced the re-epithelization and tissue granulation significantly compared with the control group. Histopathological findings showed that the healing process significantly did not fast in the mice

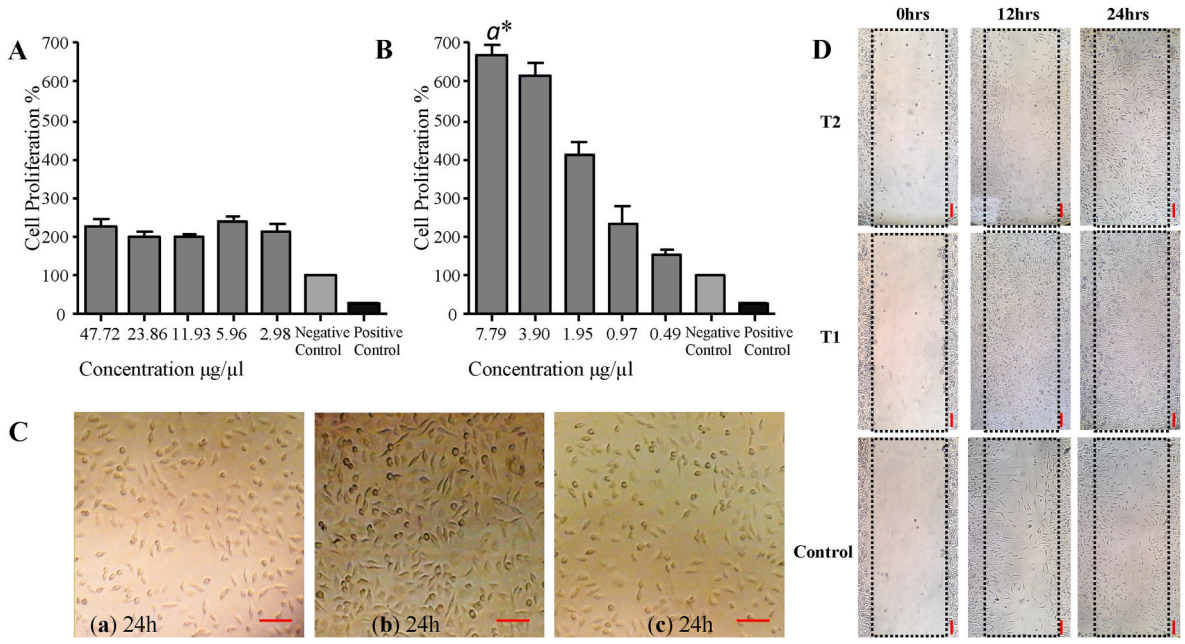


**Fig. 1.** The proliferation of mouse fibroblast cell line (L929). **A**) Treated with *Ulva lactuca* extract (2.98–47.72 µg/µl), **B**) Treated with *Sargassum ilicifolium* extract (0.49–7.79 µg/µl) and negative control were evaluated over the course of 24 h. Cell proliferation is given as a percentage of negative control cells. *a* = when compared with negative control group, (*) indicates statistically significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$). **C**) Microscopic inspection (400 × magnification) of L929 fibroblast cell after 24 h incubation with seaweeds extracts. a) negative control; b) *Sargassum ilicifolium* aqueous extracts 7.79 µg/µl; c) *Ulva lactuca* aqueous extracts 47.72 µg/µl. **D**) L929 fibroblast cell observed after injury to the cellular monolayer from the *in vitro* scratch wound healing assay. Microscopic inspection (40 × magnifications) of immediately after scratching (0 h) and after 12 and 24 h of wound healing. T1) *Sargassum ilicifolium* aqueous extracts (7.79 µg/µl) treated cells; T2) *Ulva lactuca* aqueous extracts (47.72 µg/µl) treated cells. Scale bar is 100 µm.

**Table 1**
Wound area ($mm^2$) of each test group over a period of 14 days.

| Day | Control | Treatment I | Treatment II | Treatment III |
|---|---|---|---|---|
| **0** | 63.88 ± 4.09 | 66.99 ± 3.63 | 65.66 ± 3.18 | 63.73 ± 5.15 |
| **3** | 42.21 ± 3.20 | 32.73 ± 2.83$^{ab}$* | 44.68 ± 3.77$^{a\dagger}$ | 39.00 ± 5.92 |
| **7** | 32.88 ± 1.91 | 22.27 ± 2.09$^{ab}$* | 33.54 ± 6.46$^{a\dagger}$ | 30.61 ± 4.79 |
| **10** | 19.98 ± 2.21 | 10.90 ± 1.47$^{ab}$* | 18.62 ± 1.96$^{a\dagger}$ | 16.03 ± 1.98 |
| **14** | 02.30 ± 0.27 | 0.00 ± 0.00$^{a}$* | 01.76 ± 0.41 | 00.11 ± 0.07 |

Data are expressed as values: Mean ± SE of ten replicates and analyzed by two-way analysis of variance. *$p < 0.05$ when compared with control group animals. Control is the untreated wounds. *a* = when compared with control group, *b* = when compared with treatment II Group, (*) indicates statistically significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$). (†) indicates statistically no significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$). Control: received an equal amount of distilled water, orally, Treatment I: received *S.ilicifolium* extracts (100 mg/kg BW/day for 14 days, orally), Treatment II: received *U.lactuca* extracts (100 mg/kg BW/day for 14 days, orally), Treatment III: received the topical application of the aqueous extract of *S.ilicifolium* (2 g/kg BW/day, for 14 days).

group treated with aqueous extract of *U. lactuca* group. Treatment with *S. ilicifolium* extract resulted in decreased inflammation, increasing the rate of tissue proliferation as well as remodeling, along with reepithelization. Moreover, signs of epidermal regeneration were evident in groups that were treated with *S. ilicifolium* extracts (Fig. 3).

### 3.2.2. Toxicity study

*3.2.2.1. Effect of seaweed extract on body weight of mice.* The body weight was continually measured for 28 days. In the treatment groups I, II, and III mice, body weights were not significantly different compared with control and normal mice groups.

*3.2.2.2. Serological parameters for the toxicity assessment.* An aspartate aminotransferase (AST), alanine aminotransferase (ALT), and serum creatinine levels of mice in each group are given below (Table 2).

The Biochemical parameters such as AST, ALT, and creatinine levels did not exhibit significant difference from their normal ranges (AST: 54–298 IU/L, ALT: 17–77 IU/L, Serum creatinine: 0.2–0.9 mg/dl (Laboratory mice, research animal resources, University of Minnesota) in both the seaweed extract treated groups (I, II and III) and control groups (IV and V) were comparable with each other (Fig. 4). Thus it is also found that there is a considerable variation between the two seaweeds treated mice groups and control groups. Those results indicate that the selected two seaweeds are safe for human consumption and do not indicate hepatotoxicity.

*3.2.2.3. Effect of seaweed extract on organ weight of mice.* The mean weights of all the vital organs were similar among both control and all the experimental groups. There were no significant differences ($p < 0.05$) observed in the organ weight in all the groups on the 17, 21, 24, 28 days of sacrifice. The mean weights of the organs were found to be similar (Table 3).

These results indicated that weights (g) of vital organs such as liver, kidney, heart, spleen, and lung had not been affected due to oral administration of seaweeds during the experimental period, had supported by the results of biochemical parameters and by the histopathology of different organs of mice treated orally with the aqueous extract.

*3.2.2.4. Histopathological sections of vital organs.* Histopathological sections of liver, kidney, heart, spleen, and lungs in mice of each treatment group for 14th, 17th, 21st, and 28th days exhibited normal histology compared with controls. No considerable morphological changes were observed in sections of the vital organs. In our study, the kidney showed the absence of mesangial cell hyperplasia, fibrosis, tubular ectasis, necrosis, hyperemia, glomerular basement membrane

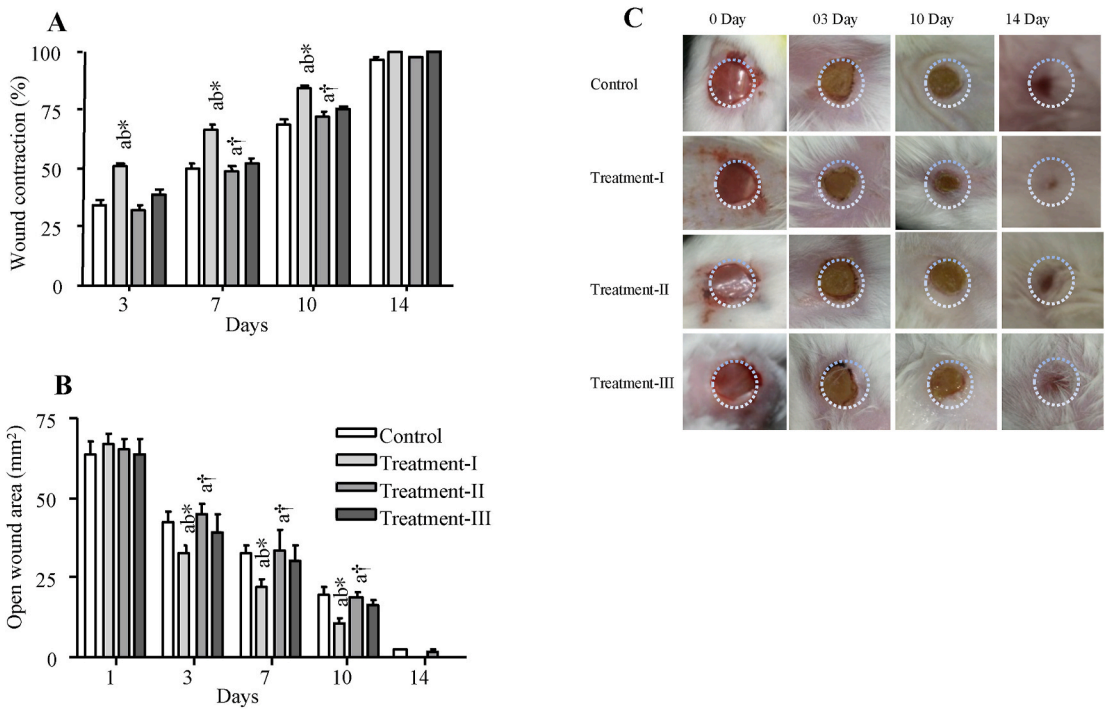


**Fig. 2.** Wound healing activity of seaweed *Sargassum ilicifolium* and *Ulva lactuca* aqueous extracts. **A**) The wound healing percentages of each test group over a period of 14 days. **B**) Changes in wound area at each time point to the original wound area of mice in each group over a period of 14 days. **C**) Digital Photographs of mice showing various stages of wound healing. Day 0 picture was taken immediately after injury. Values are diameter of wounds expressed as mean ± SE ($N = 10$). Control is the untreated wounds. $a$ = when compared with control group, $b$ = when compared with treatment II Group, (*) indicates statistically significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$). (†) indicates statistically no significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$).

thickening in mice treated with seaweed extracts. Moreover, heart cells of both treatment groups and control groups exhibited normal morphology with the absence of hypertrophy, dilation, or inflammation of cells and with the presence of an orderly array of cell nuclei. Morphology of lung cells was comparable between treatment and control groups. No considerable abnormalities, namely interstitial thickenings, pulmonary edema, pulmonary fibrosis, hemorrhage in alveolar were observed in all four groups.

### *3.2.3. Effect of seaweed extracts on hematological and serological parameters*

The study revealed a significant increase in the neutrophil and monocyte counts ($p < 0.05$) in mice treated with aqueous extracts of *S. ilicifolium* within the first seven days but *U.lactuca* did not show any significant difference with control group. Neutrophil count increases when an animal response to injuries and infections (Fig. 5). Mice treated with extracts of *S. ilicifolium* and *U. lactuca* of 100 mg/kg did not show any significant effect in total serum protein levels, serum glucose level, and packed cell volume (PCV) when compared to the control group (see Table 4).

## 4. Discussion

Wounds can disrupt skin integrity by many intrinsic and extrinsic factors such as physical, chemical, or microbial effects. Therefore, studies on wound healing agents are one of the developing areas in novel biomedical sciences. In recent years, seaweed products have been widely used as health-promoting agents, but their wound healing potentials have not yet received much attention. Therefore, using seaweed products to explore novel wound healing treatments that can speed up the healing process might be very useful. Many scientific phytochemical screening studies have revealed that certain alkaloids, triterpenoids, and flavonoids might play a major role in the process of wound healing by increasing the viability of collagen fibrils or by preventing cell damage, or by promoting DNA synthesis [21]. The brown algae are reported to have an effect on the inflammatory and immune systems [22]. It has been shown that the presence of alkaloids in the early phases of wound healing stimulate, and saponins have been shown to modulate wound cell function which can increase fibroblast proliferation and migration [23]. Cell migration and proliferation are limiting factors in skin restoration [24]. A safe treatment for wound healing should ideally have a therapeutic agent that will improve new tissue formation without producing any undesirable side effects [21].

In our study, *in vitro* experiments using aqueous extracts of *S. ilicifolium* and *U. lactuca* did not show any cytotoxic effects on the L929 cell line. The extract of *S. ilicifolium* has induced promising cell proliferation and migration activity on the L929 cell line in the scratch wound healing assay. It represents the second phase of wound healing described by the proliferation and migration of keratinocytes or fibroblasts cells. Moreover, the *in vivo* studies of wound healing, including observation of different physical, histological and biochemical parameters indicated that the aqueous extract of *S. ilicifolium* displays wound healing properties. Histopathological findings revealed that the healing process was significantly faster in the mice group treated orally with *S. ilicifolium* aqueous extract. During this study, oral administration of the extracts was performed since it is non-painful, safe, and avoids intrahepatic circulation.

The histopathological sections proved that the content of the granulation tissue of the animals treated with aqueous extract of *S. ilicifolium* was significantly increased when compared to the control and the group of animals treated with *U. lactuca*. Enhanced healing activity may also be attributed to increased collagen formation and angiogenesis [25,26]. Angiogenesis in granulation tissues improves blood supplementation to the wound site, thus providing nutrients and oxygen essential for the healing process [27]. In the current study, an increased rate of wound contraction and epithelialization in *S. ilicifolium* aqueous extract-treated

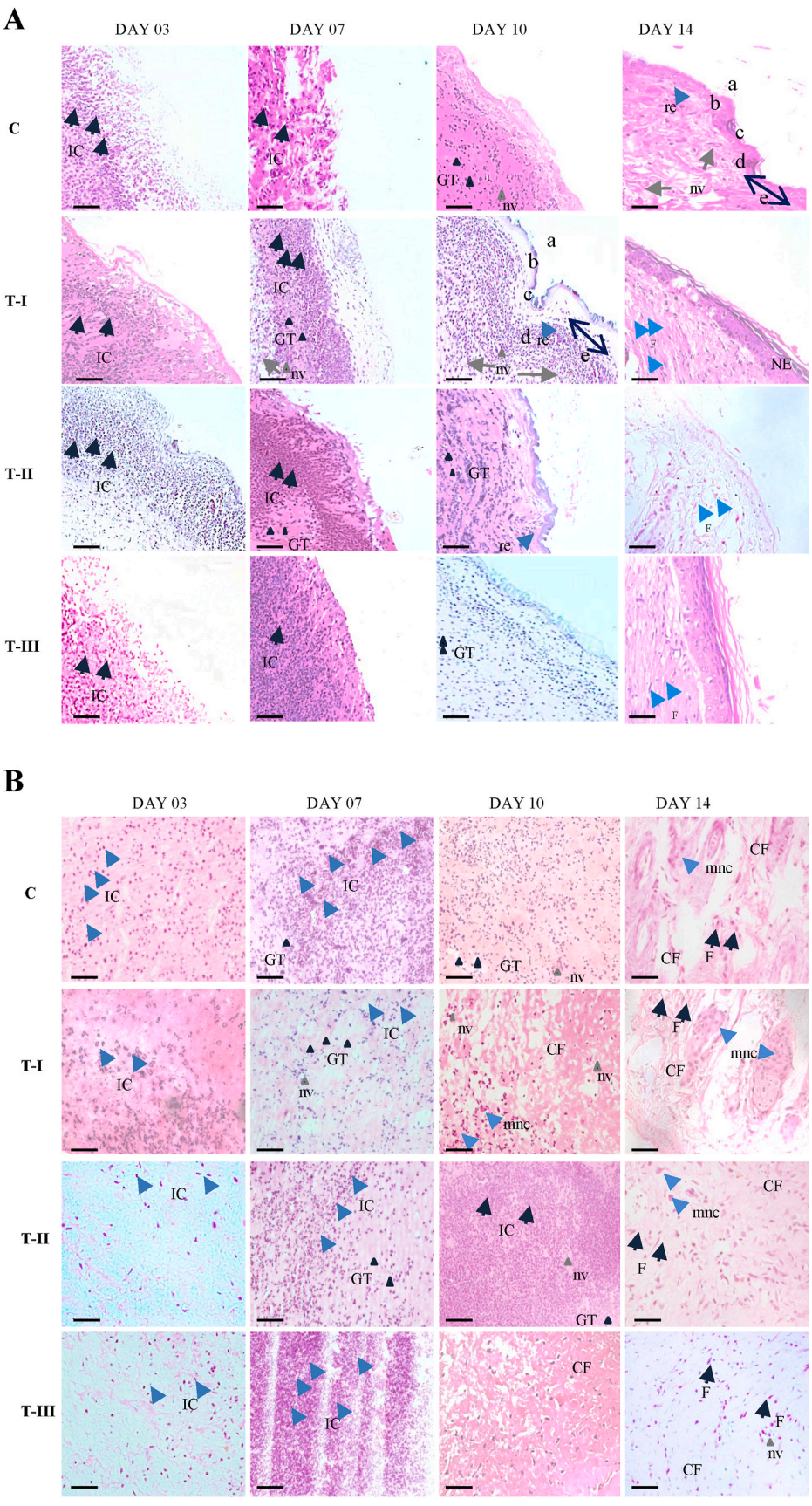


**Fig. 3.** Histological evaluation of skin wound healing. **A)** Photomicrographs of epidermis sections of wound tissues from wound area of mice stained with hematoxylin and eosin stain (H & E stain; 400 ×). **B)** Photomicrograph demonstrating granulation tissues from wound area of mice stained with hematoxylin and eosin stains (H & E stain; 400 ×). C) Control, T-I) Treatment I, T-II) Treatment II, T-III) Treatment III. Skin appears well rearranged epidermis. (a) *stratum corneum*, (b) *stratum granulosum* (c) *stratum spinosum*, (d) *stratum basale*, (e) *papillary layer*. Arrows pointing events during wound healing; re: re-epithelialization, IC: inflammatory cells, nv: neovascularization, GT: granulation tissue, F: fibroblasts, CF: collagen fiber, mnc: mononuclear, NE: new epithelium. Scale bar is 100 μm.

**Table 2**
Biochemical parameters of mice in different groups.

| Biochemical test | Normal | Control | Treatment I | Treatment II | Treatment III |
|---|---|---|---|---|---|
| AST (IU/L) | 208.4 ± 10.52 | 193.6 ± 22.92 | 170.5 ± 24.06$^{a\dagger}$ | 146.2 ± 04.29$^{a\dagger}$ | 179.2 ± 34.53$^{a\dagger}$ |
| ALT (IU/L) | 75.88 ± 19.00 | 268.8 ± 35.22 | 248.5 ± 47.82$^{a\dagger}$ | 321.9 ± 43.34$^{a\dagger}$ | 211.6 ± 29.92$^{a\dagger}$ |
| Creatinine (mg/dl) | 0.53 ± 0.01 | 0.51 ± 0.03 | 0.47 ± 0.04 | 0.54 ± 0.02 | 0.43 ± 0.03 |

Data are presented as the Mean ± SE of n = 10 mice of each experimental group and analyzed by one-way analysis of variance. *p < 0.05 when compared with control group animals. Control is the untreated wounds. *a* = when compared with control group, *b* = when compared with treatment II Group, (*) indicates statistically significant difference from respective group using ANOVA, followed by Tukey comparisons test (p > 0.05). (†) indicates statistically no significant difference from respective group using ANOVA, followed by Tukey comparisons test (p > 0.05). Control: received an equal amount of distilled water, orally, Treatment I: received *S.ilicifolium* extracts (100 mg/kg BW/day for 14 days, orally), Treatment II: received *U.lactuca* extracts (100 mg/kg BW/day for 14 days, orally), Treatment III: received the topical application of the aqueous extract of *S.ilicifolium* (2 g/kg BW/day, for 14 days).

animals could be observed. Thus, the effect of *S. ilicifolium* aqueous extract on wound contraction and epithelialization suggests it may enhance epithelial cell migration and proliferation, as well as the formation, migration, and action of myofibroblasts. To differentiate wound contraction from re-epithelialization, wound contraction was determined by the change in diameter of the whole wound. Our observations conclude that re-epithelialization plays a major role in wound healing in *S. ilicifolium* aqueous extract-treated mice. It was observed that oral application of *S. ilicifolium* extracts, enhanced cutaneous healing, in which healing was completed within 12 days. According to an investigation of blood film, within a few hours after injury, immune cells such as neutrophils, monocytes, and lymphocytes invade the wound site. Approximately after 24 h after the injury, the neutrophils come first and become the predominant leukocyte in the wound where they remove foreign material, bacteria, and wound debridement [28,29]. Followed by, monocytes count is increased to the wound site through the bloodstream. Few days after injury (2–7 days), monocytes differentiate into macrophages which - come into play in the wounded area and establish themselves as the main cell population until fibroblast migration and replication [29].

Toxicity findings of this study showed that there is no toxic effect from the *S. ilicifolium* and *U. lactuca* aqueous extracts on mice. In this study, experimental animals remained healthy with normal weight gain during the experiment period. The liver cells get damaged with a loss of the functional integrity of cell membranes as a result of the introduction of infectious agents or chemicals. With the increased damage, the serum enzyme levels, namely, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) tend to increase significantly [30,31], but such changes were not observed in the present study. Comparison of organ weights of control group and treatment group animals have been used to estimate the toxic effects widely in toxicological studies [32,33]. Similarly, feeding of *S. ilicifolium* extract did not cause gross or histopathological changes in the test groups. With the preliminary observations gained through the current study, more in-depth analyses should be conducted in future studies to elucidate the involvement of the active compounds of *S. ilicifolium* in the wound healing mechanism.

## 5. Conclusion

The present study revealed that aqueous extracts of *S. ilicifolium* have the potential to enhance wound healing activity by oral administration compared to the one from *Ulva lactuca*. It was possible to relate the favorable effect of *in vitro* and *in vivo* wound healing of the *S. ilicifolium* extracts to their effects on repair processes. Histopathological findings also supported the wound healing ability of the extracts. However,

**Table 3**
Organ weights of mice in each experimental group.

| Organ Weights (g) | Normal | Control | Treatment I | Treatment II | Treatment III |
|---|---|---|---|---|---|
| Liver | 1.439 ± 0.039 | 1.431 ± 0.044 | 1.444 ± 0.048 | 1.433 ± 0.098 | 1.535 ± 0.094 |
| Kidney | 0.437 ± 0.026 | 0.435 ± 0.027 | 0.396 ± 0.017 | 0.427 ± 0.034 | 0.421 ± 0.029 |
| Lung | 0.247 ± 0.015 | 0.251 ± 0.017 | 0.255 ± 0.021 | 0.271 ± 0.012 | 0.271 ± 0.014 |
| Heart | 0.218 ± 0.029 | 0.211 ± 0.030 | 0.160 ± 0.022 | 0.173 ± 0.031 | 0.168 ± 0.018 |
| Spleen | 0.148 ± 0.015 | 0.147 ± 0.021 | 0.165 ± 0.025 | 0.165 ± 0.011 | 0.150 ± 0.028 |

Data are presented as the Mean ± SE of n = 10 mice of each experimental groups. Normal: Normal mice; kept intact without wound and any treatment, Control: Wound were created and received an equal amount of distilled water, orally, Treatment I: Wound were created and received *S.ilicifolium* extracts (100 mg/kg BW/day for 14 days, orally), Treatment II: Wound were created and received *U. lactuca* extracts (100 mg/kg BW/day for 14 days, orally), Treatment III: Wound were created and received the topical application of the aqueous extract of *S. ilicifolium* (2 g/kg BW/day, for 14 days).

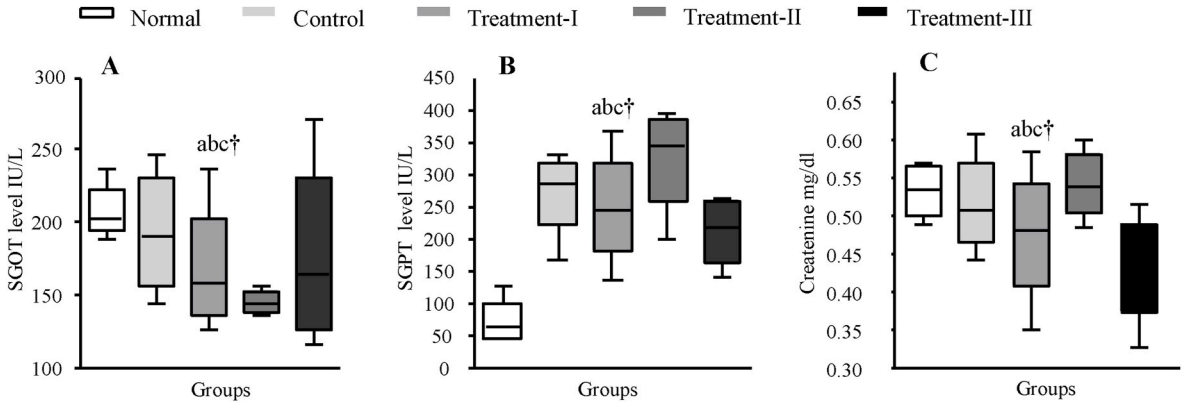


**Fig. 4.** Biochemical values of mice of each test group over a period of 28 days. A) Aspartate aminotransferase (AST), B) Alanine aminotransferase (ALT) levels in mice, C) Serum creatinine levels in mice. Values are expressed as mean ± SE; Data is compared against normal group. *a* = when compared with control group, *b* = when compared with treatment II Group, *c* = when compared with treatment III Group, (*) indicates statistically significant difference from respective group using ANOVA, followed by Tukey comparisons test (p > 0.05). (†) indicates statistically no significant difference from respective group using ANOVA, followed by Tukey comparisons test (p > 0.05).

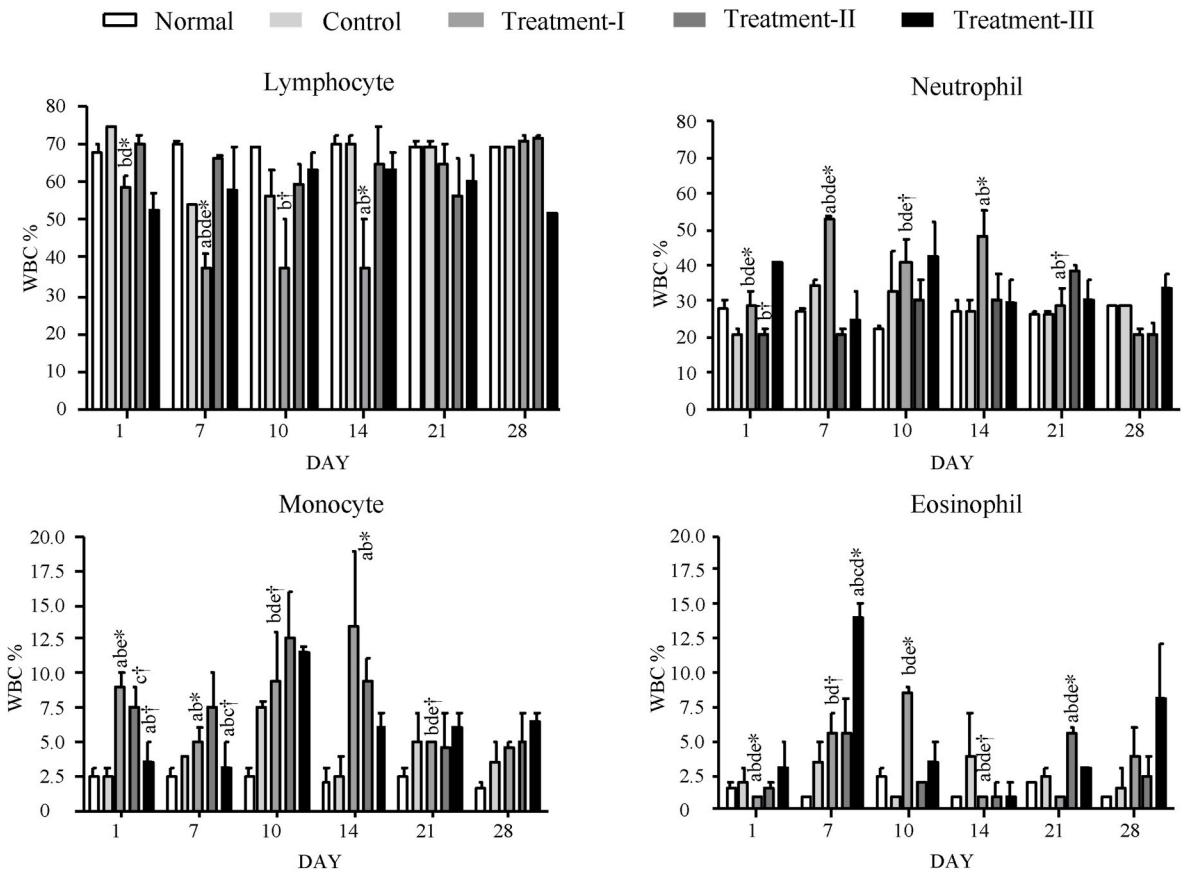


**Fig. 5.** Differential white blood cell count of mice. Values are expressed as mean ± SE; Data is compared against values in the control group. One way analysis of variance (ANOVA) Tukey-comparisons test. *$p < 0.05$. Values are expressed as mean ± SE; Data is compared against Normal group. *a* = when compared with normal group, *b* = when compared with control group, *c* = when compared with treatment I Group, *d* = when compared with treatment II Group, *e* = when compared with treatment III Group, (*) indicates statistically significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$). (†) indicates statistically no significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$).

**Table 4**
Packed cell volume, Serum protein levels and glucose level on the experiment period.

| | Normal | Control | Treatment I | Treatment II | Treatment III |
|---|---|---|---|---|---|
| **PCV** | 35.0 ± 2.73 | 29.00 ± 1.41 | 29.25 ± 3.09$^{a\dagger}$ | 28.88 ± 3.21$^{a\dagger}$ | 28.38 ± 3.30$^{a\dagger}$ |
| **Serum protein levels** | 05.87 ± 0.05 | 5.375 ± 0.30 | 05.19 ± 0.19$^{a\dagger}$ | 05.49 ± 0.18$^{a\dagger}$ | 05.37 ± 0.22$^{a\dagger}$ |
| **Glucose level** | 126.0 ± 1.48 | 130.4 ± 5.52 | 140.8 ± 6.46$^{a\dagger}$ | 142.4 ± 11.79$^{a\dagger}$ | 132.4 ± 6.28$^{a\dagger}$ |

Data are expressed as values: Mean ± SE of ten replicates and analyzed by two-way analysis of variance. *$P < 0.05$ when compared with control group animals. Control is the untreated wounds. *a* = when compared with control group, *b* = when compared with treatment II Group, (*) indicates statistically significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$). (†) indicates statistically no significant difference from respective group using ANOVA, followed by Tukey comparisons test ($p > 0.05$). Control: received an equal amount of distilled water, orally, Treatment I: received *S.ilicifolium* extracts (100 mg/kg BW/day for 14 days, orally), Treatment II: received *U.lactuca* extracts (100 mg/kg BW/day for 14 days, orally), Treatment III: received the topical application of the aqueous extract of *S.ilicifolium* (2 g/kg BW/day, for 14 days).

further investigations should be conducted to elucidate the mechanisms of action and identify the active molecules of the *S. ilicifolium* extracts. Accordingly, *S. ilicifolium* and *U. lactuca* extracts can be orally administered safely to laboratory animals or may be safely used in an experimental clinical trial.

## Ethics approval and consent to participate

Ethical clearance was obtained from the Ethical Review Committee of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka (Ref No. ERC/FVMA/UOP/2013/10), which is on par with the international standards of ethics on animal experimentations. All experimental procedures and animal care had been approved by the Faculty Ethics Committee, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka.

## Consent for publishing

We certify this manuscript has not been published elsewhere and is not submitted to another Journal. All authors have approved the manuscript and agreed with submission to the journal of "Biochemistry and Biophysics Reports".

## Availability of data and materials

The data sets and materials are contained within the paper. Animals were obtained from the Medical Research Institute (MRI) Colombo Sri Lanka.

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## Authors' contributions

AD and RPVJ conceived the study, concept, and the proposal for study design and conducted the laboratory experiments, data analyzed and interpreted experimental results and manuscript preparations. RPVJ, SK, AP, and RRMKK contributed to supervision of the study, drafting of the article. RN and RRMKK conducted all the histopathology studies; WV and RT contributed with interpretation of data and critical revision of the manuscript. AD and DL contributed with cell culture experiment and analysis. TH and NAND supported carrying out laboratory experiments. All authors read and approved the final manuscript.

## Declaration of competing interest

The author(s) declare that they have no competing interests.

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## List of abbreviations

| | |
|---|---|
| MTT | Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test |
| $CO_2$ | Carbon dioxide |
| BW | Body weight |
| RT | Room temperature |
| °C | Celsius |
| ELISA | Enzyme Linked Immunosorbent Assay |
| SE | Stranded Error |

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100986.

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