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Characterization of Alginate from *Sargassum duplicatum* and the Antioxidant Effect of Alginate–Okra Fruit Extracts Combination for Wound Healing on Diabetic Mice

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Featured Application: In this study, we evaluated antioxidant effect of alginate–okra extracts combination as topical application (ointment) for wound healing on diabetic mice. Characterization of alginate was used to determine structural characteristics and Okra fruit extract was determined total flavonoids content that have effect of the antioxidant properties. The antioxidant properties of extracts combination reduce blood-glucose levels to non-diabetic conditions (normal) significantly by topical application of diabetic open wound. These conditions can accelerate the activities of wound-healing processes on diabetic mice. The activities of wound-healing processes were performing periodically by histopathology test on skin tissue to evaluated wound healing parameters (wound area, neutrophils, macrophages, fibrocytes, fibroblasts and collagen densities).

Abstract: Diabetes mellitus is a group of metabolic disorders characterized by high blood-glucose levels over a prolonged period that causes complications when an open wound is present. Alginate is an antioxidant and a good absorbent of exudates. Okra fruit contains flavonoids that can act as antioxidants. The antioxidant properties of extracts combination reduce blood-glucose levels significantly to accelerate the activities of wound-healing processes on diabetic mice. Alginate was characterized by Size Exclusion Chromatography-Multiple Angle Laser Light Scattering (SEC-MALLS), thermal stability and Proton Nuclear Magnetic Resonance (^{1}H-NMR). The evaluation of wound healing on 36 male mice were divided into 12 groups including normal control (NC), diabetics control (DC), alginate (DA) and alginate–okra (DAO) groups in three different times by histopathology test on skin tissue. The results of SEC-MALLS analysis showed that alginate as single and homogeneous polysaccharide. The ^{1}H-NMR spectrum showed that the mannuronate/guluronate ratio of the used alginate was 0.91. Alginate, okra fruit extract and their combination were classified as moderate and strong antioxidants. The numbers of fibrocytes, fibroblasts, collagen densities had

significantly increased from three to seven days. In contrast, wound width, neutrophil, macrophages had significantly decreased at 14 days. The administration of extracts combination increased the re-epithelization of the wound area and wound-healing process on diabetic mice.

Keywords: alginate; *Sargassum duplicatum*; okra; antioxidant; diabetic; wound healing

1. Introduction

Diabetes mellitus (DM) is a disease that causes complex metabolic disorder associated with insulin resistance, impaired insulin signaling, β-cell dysfunction, abnormal glucose levels and changes in lipid metabolism [1]. The condition of high blood-glucose level (hyperglycemia) is caused by impaired of insulin production or secretion, insulin resistance. Hyperglycemia causes an increase of reactive oxygen species (ROS) levels which is not counterbalanced by an adequate supply of antioxidants. This imbalance can cause oxidative stress which is the source of the development of diabetes mellitus complications because it triggers the development of insulin resistance [2]. Complication of diabetes mellitus causes chronic wound healing disorders such as inflammation, tissue formation granulation, re-epithelization and tissue renovation [3].

All these complications often result in open wounds with long-term healing problems that frequently cause the need of amputation. Epidemiological data report that 2.8% of the world's population suffered from diabetes in 2000, and the trend is expected to increase up to 4.4% by 2030 and also the percentage of amputation in diabetic wounds is expected to increase to about 50–70% [4]. The excessive generation of ROS in diabetic conditions is caused by acute serum glucose and the accumulation of advanced glycation end-products (AGEs). Endogenous or externally supplied antioxidants (due to inadequate supply of antioxidants) can lower ROS levels through the reduction of cellular molecular oxidation. Antioxidants obtained can neutralize the oxidative stress to improve wound healing in diabetes [5].

Wound-healing processes in diabetic mice have been usually treated performed in general through the administration of topical medications, topical or systemic antibiotics and vascular reconstruction. However, open wound healing has a weakness due to the use of chemical substances that cause long-term side effects in diabetics [6,7].

Alginate is a natural polysaccharide produced from *Sargassum* sp. (among others) containing α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues [8]. Alginate—in form of hydrogels, foams, films, nanofibers, topical formulations or wafers—has been widely used as bioactive dressing for wound healing [9]. As other biocompatible materials, alginate can be loaded natural compound or drugs for improved wound-healing process like simvastatin [10], honey [11], *Aloe vera* [12], curcumin [13]. Alginate can maintain a moist condition in the wound area and acts as antibacterial and antioxidant agent, absorb wound exudate as a hemostatic agent [14–16]. Moreover, alginate with composition of guluronic acid residue (G) higher than Mannuronic acid residue (M) produce a more rigid structure, thereby increasing the availability of alginate ions to make it easier to absorb exudate and proton donor in the free radical scavenging process (antioxidant), so it is easy to wound healing. Molecular weight of polysaccharides affects the ability of radical scavenging. Reduction in molecular weight below 12 kDa can affect the conformational chain or guluronic acid content which has an impact on the availability of alginate ions in radical scavenging [17].

Abelmoschus esculentus L. is known as okra plant that contains many flavonoids that play a major role acting as antioxidant agents against ROS to inhibit free radicals [18]. Hence, the okra fruit may be used as an alternative medicine for the reduction of blood glucose and cholesterol levels as antidiabetic and antihyperlipidemic properties [19,20]. In addition, okra fruit extract can accelerate wound healing through the induction of the production of cytokine and growth factor productions [21].

In this study, we report the characterization of alginate from *Sargassum duplicatum* (J. Agardh) to evaluate its characteristic on the wound-healing process. The novelty of this study lies in investigating antioxidant effect of alginate from *S. duplicatum* and okra fruit extracts combination for wound-healing process in diabetic mice. The activities of wound-healing process were performed by using an alginate–okra extracts ointment on wound performing periodically by histopathology tests on diabetic mice skin tissues.

2. Materials and Methods

2.1. Plants Collection

Brown seaweeds of *S. duplicatum* were taken from Madura Island, Indonesia in May 2018. The species was identified at Oceanographic Research Center, LIPI, Jakarta, Indonesia (Approval Reference Number: 1/3/18-id/2018). Okra (*A. esculentus*) fruits were obtained from Rungkut district, Surabaya, Indonesia. Identification of species was conducted at the Laboratory Biosystem of Biology Department, Airlangga University, Surabaya, Indonesia.

2.2. Extraction of Sodium Alginate

The method of extraction of alginate from *S. duplicatum* referred to research from Great Hall of Products and Biotechnology of Marine and Fisheries, Jakarta with modification from LIPI. Sample of *S. duplicatum* was cleaned, dried and grinded. Two hundrd grams of dried sample was soaked using 0.1% (1:75 *w/v*) KOH for two hours, then added of 1% (1:30 *w/v*) HCl was added and let it react for an hour. The residue was then washed with water to a neutral pH and added 2% (1:30 *w/v*) Na_2CO_3 at 60–70 °C for two hours with constant stirring. The extract was added 10% of HCl until a pH in the 2.8–3.2 range was reached. Two percent Na_2CO_3 at pH 7 was added until the formation of a gel filtrate was obtained. Gel filtrate was bleached with 4% (1:2 *v/v*) NaOCl for 15 h. Filtrate was refined by using isopropyl alcohol for 30 min to form fibers [22]. The extraction of sodium alginate powder from *S. duplicatum* was pale yellow color and 12.140 ± 0.684% yield based on the dry weight of brown seaweed.

2.3. Characterization of Sodium Alginate

The characterization of sodium alginate was conducted using the SEC-MALLS for determination of molecular weight of alginate. The G/M ratio of sodium alginate from *S. duplicatum* was identified by ^{1}H-NMR spectroscopy. The thermogravimetric analysis (TGA) was performed to evaluate the strength and thermal stability of the polymer matrix by recording phase transitions and the degradation pattern of alginate based on percent of weight loss and its decomposition under nitrogen gas. All measurements were conducted at the Department of Chemistry, University of York, Heslington, UK.

2.3.1. SEC-MALLS

The molecular weight of pectin and its distribution were determined using the gel permeation chromatography technique using a Shimadzu HPLC system (Shimadzu UK Limited, Milton Keynes, UK) comprising a CBM-20A Controller, LC-20AD Pump with degasser, SIL-20A Autosampler and SPD-20A detector; and HELEOS-II light scattering and Optilab rEx refractive index detectors supplied by Wyatt. A PL Aquagel-OH mixed column (7.5 mm × 300 mm, 8-μm particle size; Agilent Technologies, Santa Clara, CA, USA) and a PL Aquagel-OH mixed guard column (7.5 × 50 mm, 8-μm particle size; Agilent Technologies, USA) were used as the stationary phase with 50-mM sodium nitrate dissolved in deionized water as mobile phase constituting. The flow rate of the mobile phase was 0.5 mL min^{-1} [23]. The alginate solution (1.5 mg mL^{-1}), dissolved in deionized water, were filtered through a nylon membrane (Whatman, UK) before analysis. For the dn dc^{-1} value, 0.168 was used [24].

2.3.2. Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR spectroscopy analysis was performed on a JEOL JNM-ECS400A spectrometer (JEOL, Peabody, MA, USA) at a frequency of 400 MHz for [1]H. D_2O was used as NMR solvent if not otherwise specified. All samples were freeze dried before analysis [25]. Acquisition parameter were: T = 90 °C and 128 scans. samples were not pre-hydrolyzed like in reference [25] since the alginate was fully soluble in D_2O.

2.3.3. Thermogravimetric Analysis (TGA)

TGA was performed on a PL Thermal Sciences STA 625 thermal analyzer (PL Thermal Sciences Limited, Surrey, UK).–10 mg of accurately weighed sample in an aluminum sample cup was placed into the furnace with a N_2 flow of 100 mL min^{-1} and heated from room temperature to 625 °C at a heating rate of 10 °C min^{-1}. From the TGA profiles the temperatures at 10% and 50% mass loss (TD_{10} and TD_{50}, respectively) were subsequently determined [26].

2.4. Extraction of Okra Fruits

Green okra fruits were cut, dried and milled until a fine powder was obtained. The sample was soaked by using 96% ethanol (1:3 (*w/v*)) and the procedure was thoroughly repeated [27]. The filtrate was evaporated under vacuum at 50 °C and then freeze dried. The sample was kept at 4 °C until further use.

2.5. Determination of Total Flavonoids Content

The total flavonoids of the okra fruits extract were determined by colorimetric aluminum chloride using UV-vis spectrophotometer [27]. Five milligrams of extract was added 5 mL 96% of ethanol until 10 mL volume. Sample solution (1 mL) was put in a 10-mL volumetric flask and added 1 mL of 10% $AlCl_3$ and 8 mL of 5% potassium acetate solution. The mixtures were incubated for 30 min at room temperature and the absorbance was determined at 431 nm. The calibration curve was made from the standard in concentrations variation of 0.01-, 0.02-, 0.03-, 0.04- and 0.05-mg/mL quercetin, respectively. The concentration variations of samples were made as same as standard of quercetin. The standard curve was plotted between concentration and absorbance, so that the equation obtained linear regression and expressed as mg quercetin equivalent (QE)/g okra fruit extract.

2.6. Antioxidant Assay

Antioxidant assay were performed on alginate and okra fruit extracts and their combination using DPPH method. The DPPH solution was prepared as 50.0-µg/mL concentration in methanol and extracts stock solutions were prepared as 1000.0-µg/mL concentration in methanol. The extract stock solutions were diluted in various of concentrations in methanol as 200.0-, 150.0-, 125.0-, 100.0-, 75.0-, 50.0-, 35.0-, 25.0-, 15.0-, 10.0- and 6.0-µg/mL concentrations. Dilution series of the samples as 100 µL and 100 µL of DPPH solution were added into 96-well plates. The mixtures were incubated in dark chamber for 30 min and was determined the absorbance at 517 nm by using an ELISA reader [28]. The radical DPPH scavenging activity was expressed as radical DPPH scavenging percentage using the formula:

$$\text{Radical DPPH scavenging } (\%) \ = \ \frac{A_{\text{DPPH}} - A_{sample}}{A_{\text{DPPH}}} \ \times \ 100\%$$

where, A_{DPPH} was absorbance of DPPH solution and A_{sample} was absorbance of DPPH solution in the presence of samples. IC_{50} values of the extracts were calculated from graphs plotting of radical DPPH scavenging (%) and concentration of the sample solutions [29].

2.7. Preparation and Induction of the Diabetic Mice

Mice (*Mus musculus*) adult male, BALB/c strains having 3–4 months of age and a body weight in the 20–35-g range were purchased from Faculty of Pharmacy, Airlangga University, Surabaya. The ethical clearance for treating animals and all the experimental protocol were approved by Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia with Approval Reference Number: 2. KE. 049.04.2019 (4 April 2019). Mice were acclimated at 12-h light and 12-h dark lighting system for two weeks. Food and beverage were guarded during research. Mice were then fed with lard for three weeks at a dose of 0.3 mL (oral) to obtain high fat diet. Mice were induced streptozotocin (STZ) using multiple low dose method 30 mg/kg body weight as much as 0.15 mL for 8 days at intraperitoneal (i.p.) to induce Type II diabetes mellitus [30]. The body weight of mice was measured before and after the administration of lard and STZ induction. Meanwhile, the blood-glucose levels were determined after STZ induction and after 3, 7 and 14 days of treatments.

2.8. Animal Grouping and Treatments

The thirty-six mice were divided into 12 groups: three and nine groups for non-diabetic as normal (N) and diabetic (D) groups, respectively. Each group contains three mice for 3, 7- and 14-day observation [24]. Non-diabetic mice were divided into 3 groups as normal control (NC_3, NC_7 and NC_{14}), while diabetic mice are divided into nine groups: three of diabetic control groups (DC_3, DC_7 and DC_{14}), three of alginate treatment groups (DA_3, DA_7 and DA_{14}) and three of alginate–okra treatment groups (DAO_3, DAO_7 and DAO_{14}) were given the Vaseline, Vaseline–alginate and Vaseline–alginate–okra ointments, respectively. The ointment was homogenized as single dose of 50 mg/kg body weight. The replication of the animal was based on the formula of Federer (1967) [31]. Mice were wounded to give 1-cm length wounds on the *glutea* (buttocks).

2.9. Wound Healing Observation

The histopathology preparation of skin tissue was conducted based on the method which was performed by the laboratory of Veterinary Pathology of Airlangga University. The histopathology test was observed in some parameters of wounds such as number of neutrophils, macrophages, fibrocytes, fibroblasts, collagen densities and cell regeneration areas (wound width) using a light microscope and the ImageJ software. Neutrophils were recognized as polymorphonuclear cells, lobulated nucleus which have small granules in their cytoplasm. Macrophages in connective tissue of healing area derived from monocytes which were identified as large, round cells with round nucleus and often have vacuolated cytoplasm because of their phagocytic activity. Fibroblasts are large flat and irregular shape cells, containing an oval prominent shaped nucleus and basophilic cytoplasm. In contrast, fibrocytes are inactive form of fibroblast which have darkly stained elongated nuclei, spindle shape and acidophilic cytoplasm.

2.10. Statistically Analysis

All the observations of wound healing were presented as a mean ± standard error mean (SEM). The statistical analysis of wound healing observations was performed using normality and homogeneity test, one-way ANOVA and Duncan test. Nonparametric of Kruskal–Wallis and Mann–Whitney test were used if the results of normality and homogeneity were not qualified ($\alpha = 0.05$). The difference between the various mean were calculated by using IBM company (Corporate headquarters 1 New Orchard Road Armonk, New York 10504-1722, United States US: 914-499-1900) with the SPSS 20.0 software.

3. Results

3.1. Characterization of Sodium Alginate

3.1.1. Analysis of SEC-MALLS

Sodium alginate from *S. duplicatum* was characterized by SEC-MALLS to determine molecular weight and polydispersity index. The average molecular weight of alginate *S. duplicatum* was determined via SEC-MALLS analysis (Figure 1a). Sodium alginate extracted from *S. duplicatum* contains polysaccharides with weight-average molecular weight (M_w) and number-average molecular weight (M_n) of 3.535×10^4 g/mol and 2.015×10^4 g/mol, respectively. Molecular weight of commercial alginate products from brown seaweed about $0.32–4.0 \times 10^5$ g/mol [32]. Alginate's dispersity (M_w/M_n) was 1.76, showing a good homogeneity of the polysaccharide alginate from *S. duplicatum*.

3.1.2. Thermogravimetric Analysis (TGA)

TGA was required to check the thermal stability and strength of the polymer matrix. TGA was used to determine the degradation temperature and the overall thermal stability of alginate. Thermal stability and strength of the polymer matrix of the alginate from *S. duplicatum* was determined via thermogravimetric analysis (Figure 1b). The 5% of the mass was lost (TD_5) at 75 °C, it indicated that water loss. The 10% of the lost mass (TD_{10}) occurred at a temperature of 140 °C. The temperature when 20% of the mass was lost (TD_{20}) occurs at 223 °C. That is indicated as the destruction of the glycosidic bond [33]. The 50% of the lost mass (TD_{50}) occurred at 380 °C. That is indicated as the material of carbon intermediates charcoal [34].
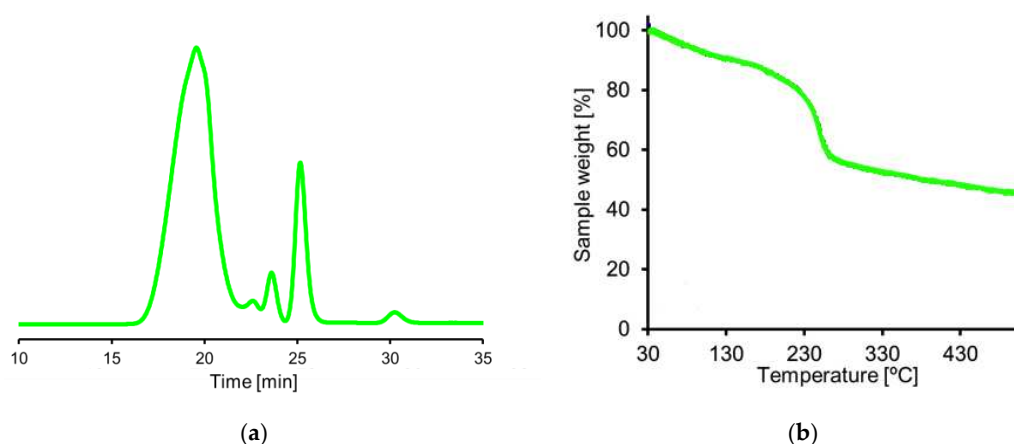

(**a**)　　　　　　　　　　　　　　　　　　(**b**)

Figure 1. (**a**) Size Exclusion Chromatography-Multiple Angle Laser Light Scattering (SEC-MALLS) profile and (**b**) TGA of the sodium alginate from *Sargassum duplicatum*.

3.1.3. Proton Nuclear Magnetic Resonance (^{1}H-NMR) Analysis

The analysis was done according to Llanes et al. [25]. ^{1}H NMR spectra analysis was used to determine the Mannuronate/Guluronate (M/G) ratio of the used sodium alginate. Quantitatively, the guluronic and mannuronic mole fractions were associated with the area signal or integration of the respective peaks. The integration obtained is shown in I_A (guluronate peak area of G_1) 5.5–5.7 ppm, I_B (mannuronate peak area of M_1) 5.1–5.4 ppm, I_C (guluronic peak area of G_5) 4.9–5.1 ppm. The mole fraction of each composition of F_M and F_G is used to determine the M/G ratio of alginate with the equation referring to the research of Grasdalen et al. [35]. The signals of proton for alginate at ^{1}H-NMR spectrum has in range of 1–6 ppm. The anomer protons of each monosaccharide could be recognized from the α-glycosides and β-glycosides of the residues. Based on the ^{1}H-NMR spectra (Figure 2b), the anomer protons of α-guluronate and β-mannuronate were shown on the G_1 and M_1 proton at δ 5.6 and 5.1 ppm, respectively. Both signals are the most deshielded proton because both protons are

attached to anomeric carbon. The signals of 5–6 ppm belong to anomeric proton of the α-glycosidic or α-guluronate and the signals between in the range of 4.3–5 ppm belong to proton of β-glycosidic or β-mannuronate [36]. The proton of α-glycosidic is more deshielded than β-glycosidic because it is located at equatorial position as compared to axial position. Llanes et al. [25] showed proton signals of sodium alginate from *Sargassum* sp. that G_1, M_1, G_5 proton at δ 5.07, 4.68 and 4.45 ppm. The M/G ratio of the mannuronic and guluronic acid residues can be determined from the integration of the respective peaks of the ^{1}H-NMR spectra [37]. The M/G ratio and η value of alginate *S. duplicatum* were 0.91 and 0.68, respectively. The chemical structure of mannuronic acid and guluronic acid could be shown in Figure 2c,d.
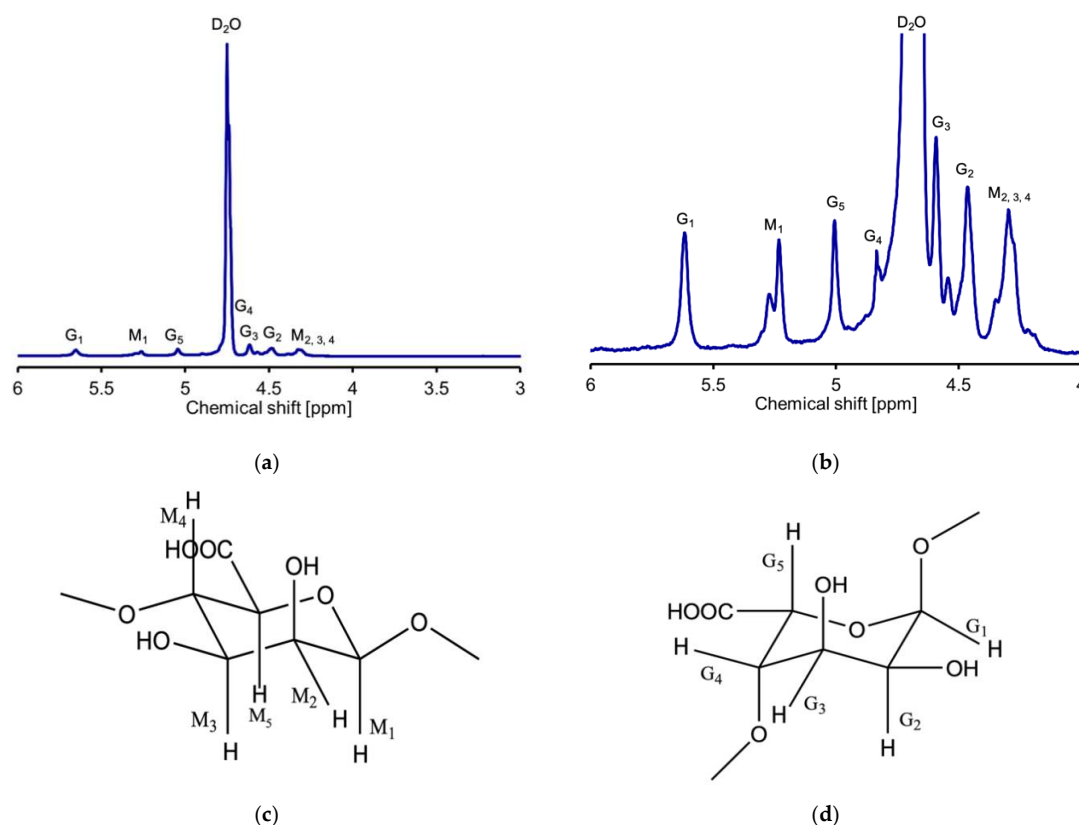

Figure 2. (**a**) ^{1}H-NMR spectra of sodium alginate from *S. duplicatum*; (**b**) details of ^{1}H-NMR spectra; (**c**) structure of mannuronic acid; (**d**) structure of guluronic acid.

3.2. Total Flavonoids Content and Antioxidant Assay

The total content of flavonoids of okra fruit extract was 43.96 mg (QE)/g or 4.40% (y = 0.0105x − 0.0161; R^2 = 0.9989). The parameter used to study the antioxidant activity is the value of inhibition concentration (IC_{50}) and it is classified in to 5 groups (very strong/highly active, strong/active, moderate, weak, inactive) [38]. In this study, ascorbic acid was selected as reference antioxidant (strong antioxidant). IC_{50} was determined using DPPH method based on the percentage of DPPH radical scavenging activity. The IC_{50} value of alginate *S. duplicatum* was 125.31-μg/mL (y = 0.2657x + 1.7814; R^2 = 0.959). Okra fruit extract and alginate–okra fruit extracts combinations (AOEs) showed strong antioxidant comparable to ascorbic acid. As reported in Table 1 the IC_{50} value of the okra fruits and of alginate–okra fruits extracts combination were 65.87-μg/mL (y = 0.097x + 43.611; R^2 = 0.9567) and 79.34-μg/mL (y = 0.3384x + 13.957; R^2 = 0.9681), respectively.

Table 1. Antioxidant activity of alginate, okra fruit and alginate–okra fruit extracts combinations (AOEs).

Sample of Extracts	IC$_{50}$ (µg/mL)	Classification
Sodium alginate *S. duplicatum*	125.31	Moderate antioxidant
Okra fruit	65.87	Strong antioxidant
Alginate–okra extracts combination	79.34	Strong antioxidant
Ascorbic acid	55.89	Strong antioxidant

3.3. Determination of Weight on Mice

The results showed that the administration of lard for 3 weeks with a dose of 0.3 mL per oral could increase the weight of the body significantly ($\alpha < 0.05$) from 27.26 ± 4.23 g to 33.25 ± 2.97 g. Obesity of mice was characterized by increased weight after the administration of lard. (Data Not Shown)

3.4. Determination of Blood-glucose Levels

Alteration in blood-glucose levels on Day 1 to 14 after STZ induction and topical treatment is shown in Table 2. STZ was able to increase blood-glucose levels > 250 mg/dL on Day 1 which showed all diabetic groups in suffering of diabetes. Alginate administration did not lead to significantly lower blood-glucose levels ($\alpha > 0.05$) from Day 1 (251.50 ± 7.78 mg/dL) until Day 7 (229.50 ± 7.78 mg/dL) but decreased significantly at Day 14 (208.00 ± 8.49 mg/dL) if compared to the first day. In contrast, the topical treatment of alginate–okra could decrease the glucose levels significantly ($\alpha < 0.05$) from Day 1 (256.50 ± 19.09 mg/dL) until Day 14 (115.00 ± 19.06 mg/dL).

Table 2. Total blood glucose of mice on Days 1, 3, 7 and 14 after treatment.

Group Treatment	Blood-glucose Level (mg/dL)			
	Day 1	Day 3	Day 7	Day 14
Normal (NC)	111 ± 9.9	110.5 ± 7.8	107.5 ± 5.0	120.0 ± 14.1
Diabetic (DC)	269.5 ± 16.3	252.5 ± 19.1	287.5 ± 14.9	220.0 ± 24.0
Alginate ointment (DA)	251.5 ± 7.8	239.5 ± 27.6	229.5 ± 7.8	208.0 ± 8.5
Alginate–okra ointment (DAO)	256.5 ± 19.1	169.5 ± 24.8	182.5 ± 13.4	115.0 ± 19.1 *

Letter notation on the table shows the result of the statistical test ($\alpha < 0.05$). (*) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \leq \alpha \leq 0.05$. *n* = 3 animals per time point.

3.5. Histological Analysis of Wound Tissue

Histological analysis of the width of the wound was indicated by yellow line an area of re-epithelization on the wound (Figure 3). The re-epithelization area increased on Day 7 for both DA and DAO and wounds closed occurs on Day 14 for each group. The DAO groups showed that complete re-epithelization and faster than other groups and proved by histology on Day 7 and 14.
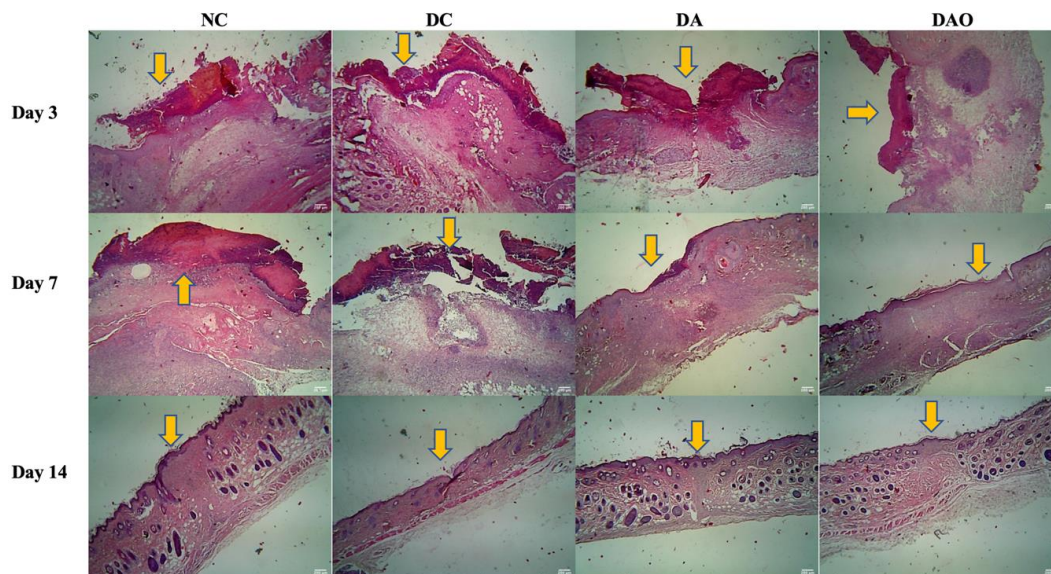
Figure 3. Histology of the wound width indicated by the yellow arrow. NC—normal control group; DC—diabetic control group; DA—alginate treatment group; DAO—treatment of alginate–okra fruit extracts. All groups have three variation times.

Wound parameter such as neutrophils (pale green), macrophages (yellow), fibrocytes (green), fibroblasts (blue) and collagen densities (orange) showed by histology magnification of wound width (Figure 4). The number of neutrophils reaches the maximum at Day 3 while it decreases from Day 3 to Day 7 and 14 for all studied groups. The number of macrophages reaches the maximum at Day 7 while it decreased at Day 14 for all groups. The DC group was evidenced by the highest concentration of macrophages when compared to other groups. Fibrocyte and fibroblast were found in the Day 7 for re-epithelization and decreased in the Day 14, as they have formed collagen fibers for all groups. The DC group was found a number of fibrocytes, fibroblasts and the least formation of collagen fibers in the group.
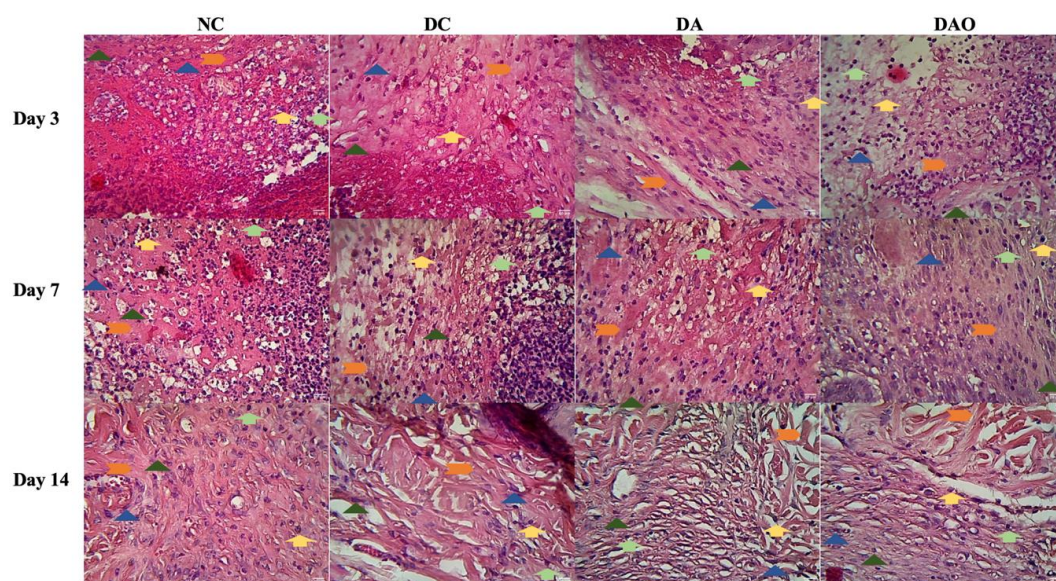

Figure 4. Histology of wound parameter including neutrophils—; Macrophages—; Fibrocytes—; Fibroblasts—; Collagen density—; NC—normal control group; DC—diabetic control group; DA—alginate treatment group; DAO—treatment of alginate–okra fruit extracts. All groups have three variation times.

3.6. Analysis of Wound Parameter

3.6.1. Wound-Width Measurement

The difference in the wound width parameter of each group for time variation is shown in Figure 5. The wound width of diabetic DC group was significantly decreased from Days 3 to 14. DC group showed significant difference to normal NC group and diabetic groups as DA, DAO treated with alginate and okra fruit extracts ointment ($\alpha < 0.05$). Treatment group of DA and DAO were significantly decrease on Days 3 to 14 and they showed significant difference on Days 3 to 7. It showed that okra fruit extracts combination plays an important role in wound closure. No significant differences between DAO group and normal group (NC) on Days 3, 7 and 14 ($\alpha > 0.05$) was observed. This indicates that the topical administration of alginate and okra fruit extracts combination can increase re-epithelization of the wound area in comparison to the control group. Diabetic group (DC) showed that still has wound width on Day 14. Otherwise, NC, DA and DAO groups showed no wound width on Day 14 and it indicated a complete wound closure.
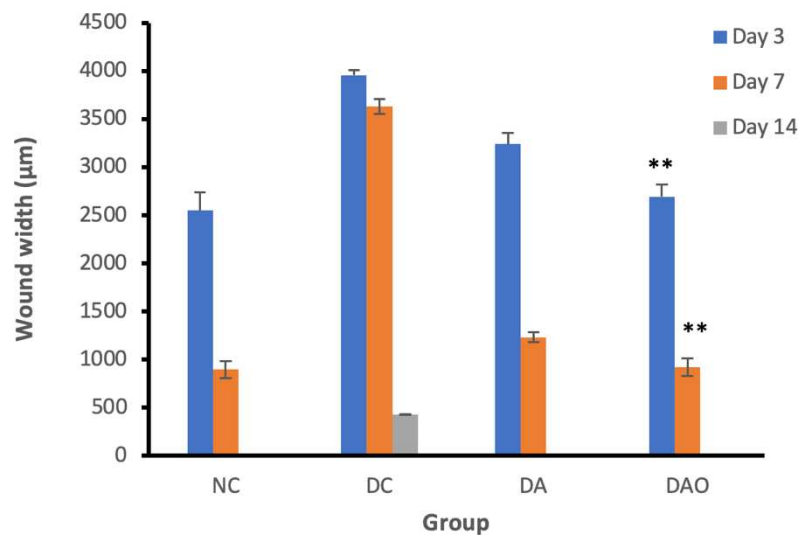

Figure 5. Graph of the wound width parameters. Notation on the graph shows the result of the statistical test ($\alpha < 0.05$). (**) notation shows insignificant differences with NC, but significant differences with DC. ** = $\alpha \leq 0.01$. NC—normal control group; DC—diabetic control group; DA—alginate treatment group; DAO—treatment of alginate–okra fruit extracts. All groups have three variation times.

3.6.2. Neutrophil Measurement

The differences in the number of neutrophils of each group in variations of time were represented in Figure 6. There were significant differences in the number of neutrophils parameters between treatment groups and DC group ($\alpha < 0.05$), but no significant differences between the treatment group and the NC group ($\alpha > 0.05$) were observed. The number of neutrophils increased on Day 3 and decreased on Day 7 and 14 for all groups.
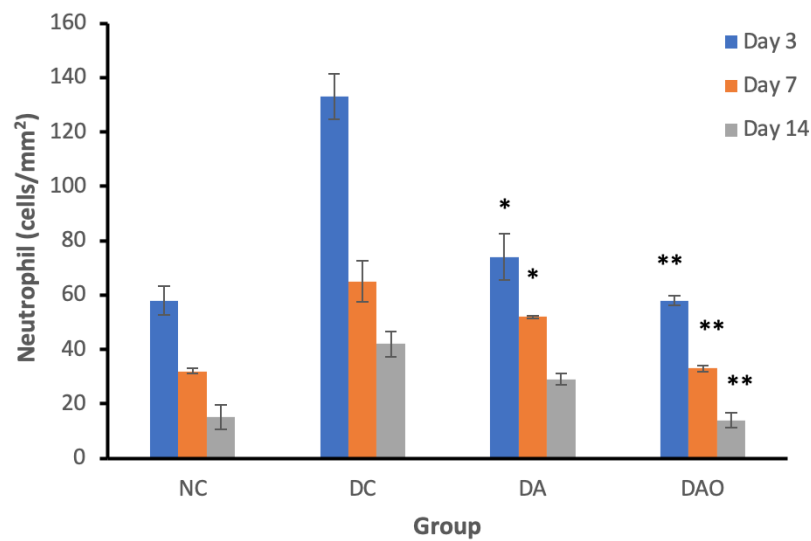
Figure 6. Graph of the number of neutrophils. Notation on the graph shows the result of the statistical test ($\alpha < 0.05$). (*) and (**) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \le \alpha < 0.05$; ** = $\alpha \le 0.01$. NC—normal control group; DC—diabetic control group; DA—alginate treatment group; DAO—treatment of alginate–okra fruit extracts. All groups have three variation times.

3.6.3. Macrophage Measurement

The difference in the number of macrophages each group in time variations were represented in Figure 7. Similar to the neutrophil parameter, the number of macrophages of DAO group was significant difference to the DC ($\alpha < 0.05$), but no significant difference with NC group ($\alpha > 0.05$). The increase of macrophages occurred up to Day 7 and decreased on Day 14 for all groups.
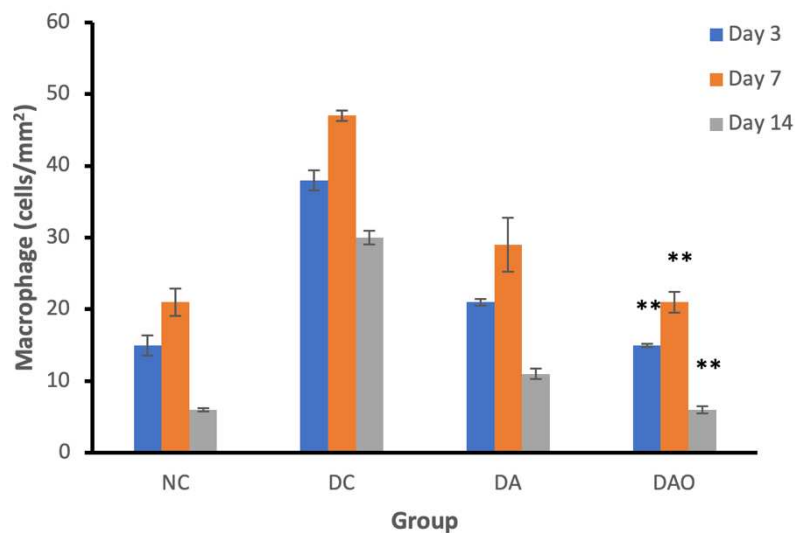

Figure 7. Graph of the number of macrophages. Notation on the graph shows the result of the statistical test ($\alpha < 0.05$). (**) notation shows insignificant differences with NC, but significant differences with DC. ** = $\alpha \le 0.01$. NC—normal control group; DC—diabetic control group; DA—alginate treatment group; DAO—treatment of alginate–okra fruit extracts. All groups have three variation times.

3.6.4. Fibrocyte and Fibroblast Measurements

The difference in the number of fibrocytes and fibroblasts of each group in time variations are shown in Figure 8a,b. There were significant differences between DAO treatment with DC groups

($\alpha < 0.05$), but no significant difference with NC group ($\alpha > 0.05$). The number of fibrocytes and fibroblasts increased on Day 7 and decreased on Day 14 for all groups.
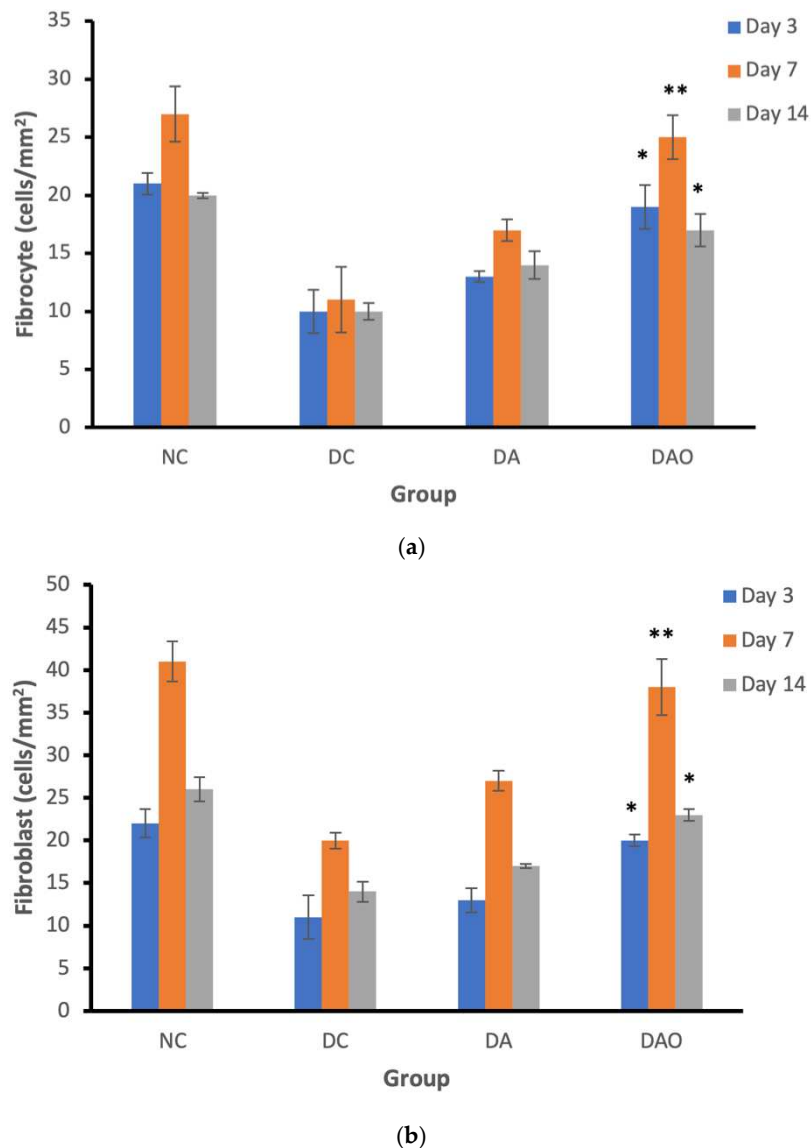

(**a**)

(**b**)

Figure 8. (**a**) Graph of the number of fibrocytes; (**b**) graph of the number of fibroblasts. Notation on the graph showed the result of the statistical test ($\alpha < 0.05$). (*) and (**) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \leq \alpha < 0.05$; ** = $\alpha \leq 0.01$. NC—normal control group; DC—diabetic control group; DA—alginate treatment group; DAO—treatment of alginate–okra fruit extracts. All groups have three variation times.

3.6.5. Collagen Densities Measurement

The different collagen densities of each group in time variations are shown in Figure 9. There were significant differences between treatment groups with DC ($\alpha < 0.05$), but no significant difference with NC group ($\alpha > 0.05$) was observed.
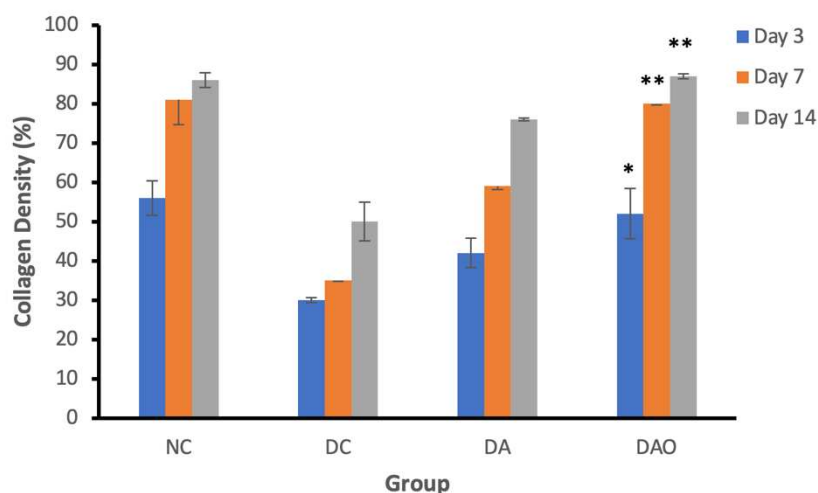
Figure 9. Graph of the collagen densities. Notation on the graph shows the result of the statistical test ($\alpha < 0.05$). (*) and (**) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \leq \alpha < 0.05$; ** = $\alpha \leq 0.01$. NC—normal control group; DC—diabetic control group; DA—alginate treatment group; DAO—treatment of alginate–okra fruit extracts. All groups have three variation times.

4. Discussion

The extraction yield of alginate from *S. duplicatum* was higher in comparison with the other seaweed such as *Cystoseira barbata*, *Dictyota caribaea* and *Padina perindusiata* of 9.9, 7.4 and 5.4%, respectively [39,40]. However, the extraction yield was lower than alginate from *C. implexa* and *L. variegate* of 29.15 and 27.57% [41], *S. angustifolium* and *Sargassum* sp. of 40.78 and 44.32%, respectively [42,43]. In general, the content of alginate in various types of brown seaweed varies widely are depending on species and the method for extraction used [44]. The extraction yield and the color of alginate from *S. duplicatum* in this study like to the previous published literature which have pale yellow color and 12% yield [40].

Based on result of characterization of alginate *S. duplicatum*, polymer of alginate *S. duplicatum* is homogeneous because it has polydispersity index is less than two [45]. M_w and M_n of alginate of *S. duplicatum* were lower in comparison to M_w and M_n of *S. turbinarioides* (5.528×10^5 and 3.852×10^5 g/mol) and *S. vulgare* (1.10×10^5 and 1.94×10^5 g/mol) [46,47]. Alginate of *S. duplicatum* has residue of guluronate higher than mannuronate (M/G ratio = 0.91) and also it has η value as 0.68 (less than 1). Hence, it indicated that alginate extracted from *S. duplicatum* is a homopolymer block containing polyguluronate type structure [47]. The M/G ratio of alginate *S. duplicatum* is higher than *S. fluitans* (0.15–0.69), but lower than *S. siliquosum* (0.70–0.94) [48]. Some other *Sargassum* species have M/G ratio about 0.8–1.4 [25].

Quantitatively, okra fruit extracts has a total flavonoid and quercetin content higher from green okra extract of 27.0 mg/100 g and 20.03 mg (QE)/100 g [49,50]. The green okra fruits contain of flavonoid such as a quercetin derivative. The primary flavonoids of okra fruits are up to 70% of the total antioxidant compounds [51]. Total phenolic or total flavonoids in okra extracts play an important role in the ability of antioxidant activity in varying percentages [14]. Antioxidant activity could be classified based on the IC_{50} values, if the values less than 50 µg/mL is very strong/highly active, 51–100 µg/mL is strong/active, 101–250 µg/mL is moderate, 251–500 µg/mL is weak and more than 500 µg/mL is inactive antioxidants [38]. Okra and AOEs have strong inhibition 65.87 and 79.34, respectively in comparison with alginate of 125.31 at the same concentration. Sodium alginate from Cystoseira barbata inhibited of 174 radical scavenging activity of DPPH [12]. It showed that the antioxidant activity of alginates from different species has quite different activities, because antioxidant activity of plant extracts is associated with flavonoid or phenolic compounds in the extract and it depends on the arrangement of structural functional groups of the extracts. In addition, the existence

of hydroxyl group, monosaccharide composition, molecular hydrogen bonds, and molecular weight affects the activity of antioxidants [52]. Some structural features of polysaccharides such as molecular weight, monosaccharide composition (block G and M), availability of hydroxyl groups, and molecular hydrogen bonds have an effect on scavenging activity. Alginate that contain block G higher than block M have an increase in antioxidant activity due to diaxial linkage in block G. It causes the obstructed rotation around the glycosidic linkage, so that it can reduce the flexibility of block G. it affects the availability of the hydroxyl group and increased proton donor capability for the free radical scavenger process in alginate molecule [47]. The addition of okra fruit extract from extracts combination on antioxidant activity showed the flavonoid content in okra fruit extract plays an important role in free radical scavenging. The reduction activity of phenolic hydroxyl groups causes flavonoids to contribute hydrogen atoms so that delocalization of phenoxy radical products occurs to protect tissue damage from ROS [53]. In this research, the addition of alginate to the extract combination reduced the IC_{50} value compared to okra fruit extracts. However, alginate can stabilize flavonoids in okra fruit extracts because G and M block residues tend to form diaxial links with intramolecular or extramolecular hydrogen bonds in more stable flavonoid compounds [54].

The administration of lard increase hyperlipidemia and insulin resistance that leads to Type 2 diabetes mellitus which was marked by obesity [30]. Onset of obesity due to the accumulation of excess fat can lead to chronic diseases and complications such as diabetes mellitus and cardiovascular disease [55]. STZ administration is expected to increase the condition of hyperglycemia (increase blood-glucose levels) and created diabetic-like syndrom [29]. The destruction of the pancreatic cells were carried out by free radical of STZ which was toxic to the insulin sensitive tissues, so that the secretion of insulin hormone was decrease [56]. Topical treatment using ointment was expected to lowering blood-glucose levels that lead to the wound healing on diabetic mice. Removal of the stratum corneum layer in the skin layer during wound formation increase skin permeability thereby allowing the release of active compounds from topical formulations in the skin layer to enter the systemic circulation. In addition, active compounds pass penetration through the transappendageal route (sweat glands, hair follicles and sebaceous glands which include open channels on the outside surface of the skin) [57,58]. Tan et al. [59] revealed that the topical application of Vicenin-2 film which is a type of flavonoid-derived glycosides from various natural plants can reduce blood-glucose levels in diabetic mice. Hence, that, lowering blood-glucose levels on diabetic mice can be influenced by topical treatment of open wounds. The combination of extracts could reduce the condition of diabetes which is demonstrated in Table 2. The value observed of DAO group at day 14 was almost the same of the control group (115.0 ± 19.1). it is indicating the potentiality of okra fruit extract in reducing blood-glucose level on diabetic mice. Antioxidant properties can neutralize free radical damage so that blood-glucose levels decreases. Okra fruit extract can control blood-glucose levels, as an effective treatment for diabetic ulcers [60].

No significant differences between DAO group (2693.0 ± 12.2; 917.0 ± 91.3; 0 ± 0 μm) and normal group (NC) (2548.0 ± 187.2; 894.0 ± 87.6; 0 ± 0 μm) on Days 3, 7 and 14 (Table 3). This indicates that the topical administration of alginate and okra fruit extracts combination can increase re-epithelization of the wound area in comparison to the control group. Diabetic group (DC) showed that still has wound width on Day 14 (424.0 ± 5.2 μm). Otherwise, NC, DA and DAO groups showed no wound width on Day 14 (0 ± 0 μm) and it indicated a complete wound closure.

Table 3. Counts considering the mean and standard deviation of wound width.

Group Treatment	Wound Width (μm)		
	Day 3	Day 7	Day 14
Normal (NC)	2548.0 ± 187.2	894.0 ± 87.6	0 ± 0
Diabetic (DC)	3956.0 ± 52.2	3631.0 ± 74.8	424.0 ± 5.2
Alginate ointment (DA)	3240.0 ± 117.5	1229.0 ± 49.9	0 ± 0
Alginate–okra ointment (DAO)	2693.0 ± 12.2 **	917.0 ± 91.3 **	0 ± 0

Letter notation on the table shows the result of the statistical test ($\alpha < 0.05$). (**) notation shows insignificant differences with NC, but significant differences with DC. ** = $\alpha \leq 0.01$.

The number of neutrophils increased on Day 3 and decreased up to Day 14 for all groups, due to migration of neutrophil achieved a maximum between Day 1 and 2. The plateaued level on Day 3 and decreases on Day 5 [61]. Neutrophil secretions in diabetic conditions tend to be high compared to the normal conditions due to high ROS/RNS levels, resulting in increased inflammation and tissue damage [62]. This is evidence by the higher number of neutrophils on Day 3 up to Day 14 in the diabetic control group (133.0 ± 8.2; 65.0 ± 7.5; 42.0 ± 4.7 cells/mm^2) compared to normal control group (58.0 ± 5.4; 32.0 ± 0.9; 15.0 ± 4.5 cells/mm^2) (Table 4).

Table 4. Counts considering the mean and standard deviation of neutrophils.

Group Treatment	Neutrophils (cells/mm^2)		
	Day 3	Day 7	Day 14
Normal (NC)	58.0 ± 5.4	32.0 ± 0.9	15.0 ± 4.5
Diabetic (DC)	133.0 ± 8.2	65.0 ± 7.5	42.0 ± 4.7
Alginate ointment (DA)	74.0 ± 8.5 *	52.0 ± 0.5 *	29.0 ± 2.1
Alginate–okra ointment (DAO)	58.0 ± 1.9 **	33.0 ± 1.2 **	14.0 ± 2.6 **

Letter notation on the table shows the result of the statistical test ($\alpha < 0.05$). (*) and (**) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \leq \alpha < 0.05$; ** = $\alpha \leq 0.01$.

Number of macrophages increase up to Day 7 and decreased on Day 14 for all groups, because on the Day 7 the wound condition undergoes inflammatory and proliferation processes. For example in the DAO group on Day 3 up to Day 7 number of macrophages increased (15.0 ± 0.2; 21.0 ± 1.4 cells/mm^2) and decreased on Day 14 (6.0 ± 0.5 cells/mm^2) (Table 5). Neutrophils were secreting cytokines and granule proteins, which modulating monocyte or macrophage extravasation, phagocytosis and ROS production. In diabetics, the condition of hyperglycemia increases the activity of macrophages, thereby increasing the levels of ROS/RNS which causes a prolonged inflammatory phase [63] In addition, hyperglycemia and oxidative stress conditions can lead to polarization and modulating macrophage dysregulation, because change epigenic code which is inhibit the wound-healing process [64]. This is evidence by the higher number of macrophages on Day 3 up to Day 14 in the diabetic control group (38.0 ± 1.4; 47.0 ± 0.7; 30.0 ± 0.9 cells/mm^2) compared to normal control group (15.0 ± 1.4; 21.0 ± 1.9; 6.0 ± 0.2 cells/mm^2).

Table 5. Counts considering the mean and standard deviation of macrophages.

Group Treatment	Macrophages (cells/mm^2)		
	Day 3	Day 7	Day 14
Normal (NC)	15.0 ± 1.4	21.0 ± 1.9	6.0 ± 0.2
Diabetic (DC)	38.0 ± 1.4	47.0 ± 0.7	30.0 ± 0.9
Alginate ointment (DA)	21.0 ± 0.5	29.0 ± 3.8	11.0 ± 0.7
Alginate–okra ointment (DAO)	15.0 ± 0.2 **	21.0 ± 1.4 **	6.0 ± 0.5 **

Letter notation on the table shows the result of the statistical test ($\alpha < 0.05$). (**) notation shows insignificant differences with NC, but significant differences with DC. ** = $\alpha \leq 0.01$.

The number of fibrocytes and fibroblasts increased on Day 7 and decreased on Day 14 for all groups, because the wound conditions have peak proliferation phases for re-epithelization and angiogenesis processes [65]. For example in the DAO group on Day 3 up to Day 7 number of fibrocyte increased (19.0 ± 1.9; 25.0 ± 1.9 cells/mm^2) and decreased on Day 14 (17.0 ± 1.4 cells/mm^2) (Table 6). The same thing happened to the number of fibroblasts in the DAO group on Day 3 up to Day 7 increased (20.0 ± 0.7; 38.0 ± 3.3 cells/mm^2) and decreased on Day 14 (23.0 ± 0.7 cells/mm^2) (Table 7). Macrophages perform cytokine and growth factor secretions in the wound area to induce fibroblast migration and proliferation, production of granulation tissues, transient extracellular matrix and angiogenesis in the healing process. Conditions of high blood-glucose levels lead to migration and fibrocyte proliferation disorders, fibroblasts and keratinocytes, resulting in decreased secretion of cytokine, growth factor and extracellular matrix [63]. This is evidence by the lower number of fibrocytes on Day 3 up to Day 14 in the diabetic control group (10.0 ± 1.9; 11.0 ± 2.8; 10.0 ± 0.7 cells/mm^2) compared to normal control group (21.0 ± 0.9; 27.0 ± 2.3; 20.0 ± 0.2 cells/mm^2). The same thing happened to the number of fibroblasts on Day 3 up to Day 14 in the diabetic control group (11.0 ± 2.6; 20.0 ± 0.9; 14.0 ± 1.2 cells/mm^2) compared to normal control group (22.0 ± 1.6; 41.0 ± 2.4; 26.0 ± 1.4 cells/mm^2).

Table 6. Counts considering the mean and standard deviation of fibrocytes.

Group Treatment	Fibrocytes (cells/mm^2)		
	Day 3	Day 7	Day 14
Normal (NC)	21.0 ± 0.9	27.0 ± 2.3	20.0 ± 0.2
Diabetic (DC)	10.0 ± 1.9	11.0 ± 2.8	10.0 ± 0.7
Alginate ointment (DA)	13.0 ± 0.5	17.0 ± 0.9	14.0 ± 1.2
Alginate–okra ointment (DAO)	19.0 ± 1.9 *	25.0 ± 1.9 **	17.0 ± 1.4 *

Letter notation on the table shows the result of the statistical test ($\alpha < 0.05$). (*) and (**) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \leq \alpha < 0.05$; ** = $\alpha \leq 0.01$.

Table 7. Counts considering the mean and standard deviation of fibroblasts.

Group Treatment	Fibroblasts (cells/mm^2)		
	Day 3	Day 7	Day 14
Normal (NC)	22.0 ± 1.6	41.0 ± 2.4	26.0 ± 1.4
Diabetic (DC)	11.0 ± 2.6	20.0 ± 0.9	14.0 ± 1.2
Alginate ointment (DA)	13.0 ± 1.4	27.0 ± 1.2	17.0 ± 0.2
Alginate–okra ointment (DAO)	20.0 ± 0.7 *	38.0 ± 3.3 **	23.0 ± 0.7 *

Letter notation on the table shows the result of the statistical test ($\alpha < 0.05$). (*) and (**) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \leq \alpha < 0.05$; ** = $\alpha \leq 0.01$.

Fibroblast proliferation induces collagen synthesis and macromolecular matrix for structural formation of connective tissues. Diabetes conditions increase the activity of macrophages thereby increasing the production of ROS/RNS and lowering collagen synthesis [66]. This is evidence by the lower collagen densities on Day 3 up to Day 14 in the diabetic control group (30.0 ± 0.6; 35.0 ± 0.1; 50.0 ± 4.9%) compared to normal control group (56.0 ± 4.3; 81.0 ± 6.4; 86.0 ± 1.9%) (Table 8).

Table 8. Counts considering the mean and standard deviation of collagen densities.

Group Treatment	Collagen Densities (%)		
	Day 3	Day 7	Day 14
Normal (NC)	56.0 ± 4.3	81.0 ± 6.4	86.0 ± 1.9
Diabetic (DC)	30.0 ± 0.6	35.0 ± 0.1	50.0 ± 4.9
Alginate ointment (DA)	42.0 ± 3.8	59.0 ± 0.8	76.0 ± 0.3
Alginate–okra ointment (DAO)	52.0 ± 6.4 *	80.0 ± 0.3 **	87.0 ± 0.6 **

Letter notation on the table shows the result of the statistical test ($\alpha < 0.05$). (*) and (**) notation shows insignificant differences with NC, but significant differences with DC. * = $0.01 \leq \alpha < 0.05$; ** = $\alpha \leq 0.01$.

The topical administration of alginate can reduce the wounds width and increase cytokines and growth factor. This is because alginate has a hydroxyl group that plays a role in the properties of hydrophilicity, so that alginate can absorb moderate or heavy exudate liquid to reach 15 to 20 times in dry or gel form, so it is called a hemostatic agent [67]. Absorption of exudate on the wound area was occur due to the exchange of ions between exudate and alginate that keeps the moist condition of the wound physiologically and minimizing bacterial infections. In addition, it allows enough oxygen exchange to accelerate the formation of tissue granulation and re-epithelization [68]. The proportion of M/G residue affects alginate's ability as absorber. Higher G content is not firmly tied to molecules so it will increase the process of ion exchange between alginate and exudate wounds [69]. Based on literature, the wound healing activity of alginate topically on diabetic open wounds form complete epithelization on Day 14 [70].

The topical administration of okra fruit extract can reduce blood-glucose levels and improve wound-healing process, because it has strong antioxidant properties due to the role of the 3-hydroxyl group on the C ring of the flavonoids compounds to capture free radicals due to glucose oxidation (hyperglycemia). Decreasing ROS/RNS can reduce hyperglycemia and increase glucose metabolism, thereby lowering oxidative stress and insulin resistance which increase wound healing [71]. The ointment formula contains flavonoids could be released into wounds and then to the bloodstream so as to induce secretion of cytokine, growth factor and increase insulin production or insulin sensitivity of somatic cells. Flavonoids also cause re-formation of antioxidants and serum lipid profiles in diabetic rats [59]. The wound healing activity of the okra fruit extract topically on open wounds effectively forms the complete epithelization on 15 day [72] and achieves a complete epithelization on 16 day [73].

5. Conclusions

Extraction and characterization of alginate from *S. duplicatum* and okra fruit extracts were carried out to determine the potentiality of them as an absorber of the wound and their antioxidant activity. The novelty of this research lies in the healing activity of open wounds from a combination of alginate and okra fruit extracts. Topical formulations are more effective when alginates are combined with okra fruit extracts because of the alginate can stabilize flavonoids, so it increased wound absorber and their antioxidant properties. The results show an improvement of the re-epithelization of the wound area, the recruitment of neutrophil, macrophages, fibrocytes, fibroblasts and increased collagen densities as parameters that plays an important role in the wound-healing process.

Further research and a more detailed analysis of the characterization of alginate structures to determine the role of alginate as an antioxidant agent in the combination of topical formulations for open wounds healing are needed. Moreover, variations of doses need to be done for further research to determine the optimum dose of the extract combination in open wound healing of diabetic mice. Hence, it would help to provide more insight of the mechanism of action this combination extracts on the process of diabetic wound healing.

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