2020-12

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Submission date: 24-May-2021 10:22PM (UTC+0800)
Submission ID: 1593179890
File name: 2020-12.pdf (201.61K)
Word count: 4552
Character count: 26402

A Novel Therapeutic effects of *Sargassum ilicifolium* Alginate and Okra (*Abelmoschus esculentus*) Pods extracts on Open wound healing process in Diabetic Mice

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ABSTRACT:

This study aims to evaluate the effects of alginate extract from *Sargassum ilicifolium* and okra (*Abelmoschus esculentus*) pods extract on the re-epithelialization rate of the wound area and the number of neutrophils, macrophages, fibroblasts, fibrocytes, and collagen density in streptozotocin-induced diabetic mice. This study was done *in vivo* using male mice (strain BALB/C, 3 to 4 months old, 30 to 40g). The normal control group (KN) and the diabetic groups (induced by streptozotocin). The diabetic group was divided into 9 groups: three diabetic control groups (KD), three alginate treatment groups (KAI), and three treatment groups of alginate-okra (*Abelmoschus esculentus*) pods extract (KAIO). The treatment dose was 50 mg/kg body weight. Measurement of fasting blood sugar levels was done before and after streptozotocin injection. The treatment was carried out in three different time periods: 3 days, 7 days, and 14 days. Then, mice skin was cut and proceed for histopathological analysis. Interestingly, we indicated that the administration of okra (*Abelmoschus esculentus*) pods extract combined with alginate from *Sargassum ilicifolium* could increase the rate of re-epithelialization of the wound area, increase the number of neutrophils, macrophages, fibroblasts, fibrocytes, as well as increase synthesized collagen. The combined extract was able to improve the healing process of open wounds in diabetic mice. Therefore, it can be concluded that administration of alginate from *Sargassum ilicifolium* and okra (*Abelmoschus esculentus*) pods extract was able to improve open wounds healing in diabetic mice due to the role of antioxidant from polysaccharides and quercetin which acted as anti-hyperglycemic to anti-inflammatory properties.

KEYWORDS: *Abelmoschus esculentus*, Antioxidant, Diabetic Mice, *Sargassum ilicifolium*, Wound Healing.

INTRODUCTION:

Diabetes mellitus (DM) is a chronic disease characterized by high blood glucose levels due to glucose metabolism disorder in the body. Type-2 DM is the most common type of DM worldwide, with a world prevalence of 7.7% or 439 million population by the end of 2030. This condition is expected to increase by 69% in developing countries[1]. According to Husen *et al*. (2019), obesity is one of the main causes of DM due to increase cholesterol level in the blood that causes advance production of peroxide by mitochondria, as well as the higher risk of cell exposure to reactive oxygen species (ROS)[2]. The increase of superoxide production will increase nitric oxide (NO). These conditions will produce reactive nitrogen species (RNS) which will oxidize sulfhydryl protein groups, increase lipid peroxidation and amino acids, as well as damage the DNA, thereby endangering normal cells[3,4,5]. The present of wounds creates a more complex infection in diabetic patient[6]. It is commonly associated with macrovascular and microvascular complications. These complications are caused by several metabolism disorders and insulin dysfunction[7,8].

DM is metabolic disorders occurs due to the increased of oxidative stress (ROS), protein kinase C (PKC), and receptors for activation of end-glycation products (RAGE)[9]. Prolonged disruption of glucose metabolism will cause hyperglycemia which can impact various reactions between glucose and other molecules in cells that lead to high formation of oxidants in the body[10]. Recent therapeutic strategies, such as antioxidant agents or statin treatment in the pre-diabetic phase in diabetic patients can prevent the disruption of diabetic wounds healing process[11]. An increase in ROS that exceeds the antioxidant capacity of cells causes cell oxidative stress, thus inhibit the process of wound healing. The wound healing in diabetics could also stop due to several other factors, including specific metabolic deficiencies, physiological responses disruption such as hypoxia, red blood cell membrane changes, and blood vessel constriction[12]. Oxidative stress occurs because of an imbalance between the number of reactive molecules (ROS and RNS) against endogenous antioxidants[13].

Antioxidant is a compound that can prevent the damage of cellular components caused by chemical reactions involving free radicals[14,15]. Antioxidants is functioned by breaking chains or stabilizing molecules. One of the antioxidants used to overcome free radicals is quercetin[2]. Quercetin is a flavonoid compound contained in the seeds of okra (*Abelmoschus esculentus*). Quercetin compounds have a significant ability to reduce ROS, hydrogen peroxide, and protein oxidation by donating hydrogen atoms and stabilizing the free radicals resonantly which do not easily participate in other radical reactions[16,17]. Okra (*Abelmoschus esculentus*) plants and *Sargassum* have high antioxidant potential against free radicals caused by diabetic condition[5,18]. Okra (*Abelmoschus esculentus*) has the ability to control the condition of hyperglycemia by inhibiting the expression of α-glucosidase[2,19]. The alginate of *Sargassum ilicifolium*, which is rich in polyphenol, is able to control blood glucose level in healthy adults[20]. Therefore, this study aims to determine the effect of alginate from *Sargassum ilicifolium* and okra (*Abelmoschus esculentus*) pods extract on the re-epithelialization rate of the wound area and the number of neutrophil cells, macrophages, fibroblasts, fibrocytes, and collagen density in streptozotocin-induced diabetic mice.

MATERIAL AND METHODS:

Plant identification:

Taxonomic identification of the okra (*Abelmoschus esculentus*) and *Sargassum ilicifolium* was carried out by the Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia.

Alginate extraction from *Sargassum ilicifolium*:

Alginate extraction procedures were referred to the Jakarta Center for Marine and Fisheries Product Processing and Biotechnology Research. Fresh *Sargassum ilicifolium* (50g) was rinsed with water and added with 1,125mL of 0.1% KOH[21]. Then, 1% HCL was added at a ratio of 1:30 w/v for 1 hour and filtered. The precipitated gel was then added with 2% Na_2CO_3 and 4% NaOCl. The extract was blanched with a 2-propanol solution and dried under the sun for 12 hours. The dried fiber was smoothened and weighed.

Okra (*Abelmoscus esculentus*) pods extraction:

Okra (*Abelmoschus esculentus*) pods used were all parts of the pods. Fresh okra (*Abelmoschus esculentus*) pods (4kg) was rinsed with water, blended and macerated with ethanol. Then, the solvent was evaporated with a rotary vacuum evaporator at 50°C. The crude extract was collected, lyophilized, and weighed[2].

Acute toxicity study:

Mice given okra (*Abelmoschus esculentus*) pods extract were tested for acute toxicity based on study by Husen *et al*. (2019)[2]. The dose of 50mg/kg body weight has been proven as safe for experimental animals' administration.

Experimental animal and ethical clearance:

This study used healthy adult male mice (*Mus musculus*), strain BALB/C, with the age ranged from 3-4 months old, body weight ranged from 30-40g, obtained from the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. Mice were acclimatized for 2 weeks to provide conditions that were similar to the conditions of the Animal Laboratory, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia. All body weight and blood glucose levels were recorder before and after the administration of alginate and streptozotocin. All mice were in control of environmental conditions (25±5°C), humidity of 50±10% and 12 light/dark cycle). Mice were fed with standard pellet and drink (*ad libitum*). All treatment procedures have been tested through ethical clearance at the Animal Care and Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (Approval Reference Number: 2.KE.049.04.2019).

Experimental design:

The research was carried out at the Animal Laboratory and the Histology Laboratory of the Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia using a completely randomized design. For induction of diabetic mice, this study used streptozotocin. This study was done *in vivo* using male mice (strain BALB/C, 3 to 4 months old, 17g to 40g). The normal control group (KN) and the diabetic groups (induced by streptozotocin). The diabetic group was divided into 9 groups: three diabetic control groups (KD), three alginate treatment groups (KAI), and three treatment groups of alginate-okra (*Abelmoschus esculentus*) pods extract (KAIO). The treatment dose was 50mg/kg body weight. Only mice with fasting blood glucose levels of more than 130mg/dL were used as the diabetes group. Each group was injured by giving 1 cm glutea section and this activity was counted as day 0. The treatment was carried out in 3 different time periods: 3 days, 7 days, and 14 days. Then, mice were sacrificed. The skin was cut and proceed for histopathological analysis.

Statistical analysis:

Histopathological examination was performed using ImageJ software to evaluate the distance of wound healing. Statistical analysis was performed using SPSS software (Statistical Package and Service Solutions) version 17.0. The data with normal distribution and homogeneity of variance analyzed using ANOVA test and followed by Duncan test. Brown-Forsythe test was executed for normally distributed data and non-homogeneity of variance and then followed by t-test. The Games-Howell post-hoc test was conducted to determine the differences between the two groups.

RESULTS AND DISCUSSION:

Indonesia is an archipelago with approximately 17,508 islands and is covered by tropical rain forest, seasonal forest, swamp, subalpine shrub vegetation, coastal vegetation, and mountain vegetation. With its reflective mixture of Asian and Australian native species, Indonesia is stated to possess the second largest biodiversity in the world, with around 40,000 endemic plant species including 6,000 medicinal plants[22]. Consequently, Indonesia is rich in medicinal plants which were used by its population traditionally from generation to generation in curing diseases[4,23,24]. In addition, in this study we found the lethal dose 50% (LD_{50}) and the antioxidant activity of okra (*Abelmoschus esculentus*) was conducted *in vitro* using DPPH method. Based on Fig.1, the antioxidants contained in okra (*Abelmoschus esculentus*) pods extract have an IC_{50} value of 65.86 (y=0.097x+43.61) so that it is classified as a strong antioxidant.

Fig. 1. IC_{50} values of DPPH test for okra (*Abelmoschus esculentus*) pods extract. Quercetin compounds found in okra (*Abelmoschus esculentus*) pods extract have strong level of antioxidant.

Fig. 2. The effect of okra (*Abelmoschus esculentus*) pods extract combined with *Sargassum ilicifolium* alginate on wound width.

The groups consisted of KN3: normal control group on 3rd day; KN7: normal control group on the 7th day; KN14: normal control group on the 14th day; KD3: diabetic control group on the 3rd day; KD7: diabetic control group on the 7th day; KD14: diabetic control on the 14th day; PAI3: *Sargassum ilicifolium* alginate treatment on the 3rd day; PAI7: *Sargassum ilicifolium* alginate treatment on the 7th day; PA114:

Sargassum ilicifolium alginate treatment on the 14th day; PAIO3: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 3rd day; PAIO7: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 7th day; PAIO14: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 14th day. The different letters show significant differences (α<0.05).

Fig. 3. The effect of okra (*Abelmoschus esculentus*) pods extract combined with *Sargassum ilicifolium* alginate on neutrophils.
The groups consisted of KN3: normal control group on 3rd day; KN7: normal control group on the 7th day; KN14: normal control group on the 14th day; KD3: diabetic control group on the 3rd day; KD7: diabetic control group on the 7th day; KD14: diabetic control on the 14th day; PAI3: *Sargassum ilicifolium* alginate treatment on the 3rd day; PAI7: *Sargassum ilicifolium* alginate treatment on the 7th day; PA114: *Sargassum ilicifolium* alginate treatment on the 14th day; PAIO3: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 3rd day; PAIO7: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 7th day; PAIO14: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 14th day. The different letters show significant differences (α<0.05).

Fig. 4. The effect of okra (*Abelmoschus esculentus*) pods extract combined with *Sargassum ilicifolium* alginate on macrophages. The groups consisted of KN3: normal control group on 3rd day; KN7: normal control group on the 7th day; KN14: normal control group on the 14th day; KD3: diabetic control group on the 3rd day; KD7: diabetic control group on the 7th day; KD14: diabetic control on the 14th day; PAI3: *Sargassum ilicifolium* alginate treatment on the 3rd day; PAI7: *Sargassum ilicifolium* alginate treatment on the 7th day; PA114: *Sargassum ilicifolium* alginate treatment on the 14th day; PAIO3: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 3rd day; PAIO7: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 7th day; PAIO14: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 14th day. The different letters show significant differences (α<0.05).

Fig. 5. The effect of okra (*Abelmoschus esculentus*) pods extract combined with *Sargassum ilicifolium* alginate on fibrocytes.
The groups consisted of KN3: normal control group on 3rd day; KN7: normal control group on the 7th day; KN14: normal control group on the 14th day; KD3: diabetic control group on the 3rd day; KD7: diabetic control group on the 7th day; KD14: diabetic control on the 14th day; PAI3: *Sargassum ilicifolium* alginate treatment on the 3rd day; PAI7: *Sargassum ilicifolium* alginate treatment on the 7th day; PA114: *Sargassum ilicifolium* alginate treatment on the 14th day; PAIO3: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 3rd day; PAIO7: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 7th day; PAIO14: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 14th day. The different letters show significant differences (α<0.05).

Fig. 6. The effect of okra (Abelmoschus esculentus) pods extract combined with *Sargassum ilicifolium* alginate on fibroblasts.
The groups consisted of KN3: normal control group on 3rd day; KN7: normal control group on the 7th day; KN14: normal control group on the 14th day; KD3: diabetic control group on the 3rd day; KD7: diabetic control group on the 7th day; KD14: diabetic control on the 14th day; PAI3: *Sargassum ilicifolium* alginate treatment on the 3rd day; PAI7: *Sargassum ilicifolium* alginate treatment on the 7th day; PA114: *Sargassum ilicifolium* alginate treatment on the 14th day; PAIO3: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 3rd day; PAIO7: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 7th day; PAIO14: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 14th day. The different letters show significant differences (α<0.05).

Fig. 7. The effect of okra (*Abelmoschus esculentus*) pods extract combined with *Sargassum ilicifolium* alginate on collagen density. The groups consisted of KN3: normal control group on 3rd day; KN7: normal control group on the 7th day; KN14: normal control group on the 14th day; KD3: diabetic control group on the 3rd day; KD7: diabetic control group on the 7th day; KD14: diabetic control on the 14th day; PAI3: *Sargassum ilicifolium* alginate treatment on the 3rd day; PAI7: *Sargassum ilicifolium* alginate treatment on the 7th day; PA114: *Sargassum ilicifolium* alginate treatment on the 14th day; PAIO3: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 3rd day; PAIO7: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 7th day; PAIO14: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 14th day. The different letters show significant differences (α<0.05).

Histopathological reading of the wound healing parameters is presented in Fig. 8, which shown a significant difference among the normal, diabetic, and treatment groups. All PAIO groups had the best positive response compared to diabetic control group. The polysaccharide polymer in alginate plays role as absorbent, analgesic, anti-inflammatory, fibrinolysis, immune modulator, and has the ability to maintain homeostatic condition of wound[25,26]. The second parameter observed was the number of inflammatory cells (neutrophils), which shown a significant difference between the treatment group and the diabetic control group. The number of neutrophil cells will increase in the first 24-48 hours after the injury, then it will decrease after 7-14 days[27]. However, in diabetic control group, the number of neutrophils is relatively higher compared to other groups. The presence of high ROS levels caused a permanent hypoxic condition in the wound area.

In addition, the number of neutrophil and macrophage cells in the treatment group is also significantly different from the diabetic control group.

Fig. 8. Histopathology analysis of wound widths. Black arrow lines represent the width. a: wound widths; b: macrophages; c: neutrophils; d: fibrocytes; and e: fibroblast.
The groups consisted of KN3: normal control group on 3rd day; KN7: normal control group on the 7th day; KN14: normal control group on the 14th day; KD3: diabetic control group on the 3rd day; KD7: diabetic control group on the 7th day; KD14: diabetic control on the 14th day; PAI3: *Sargassum ilicifolium* alginate treatment on the 3rd day; PAI7: *Sargassum ilicifolium* alginate treatment on the 7th day; PA114: *Sargassum ilicifolium* alginate treatment on the 14th day; PAIO3: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the

3rd day; PAIO7: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 7th day; PAIO14: treatment of alginate-extract of okra (*Abelmoschus esculentus*) pods on the 14th day. The different letters show significant differences (α<0.05).

The group that given alginate-okra (*Abelmoschus esculentus*) has the closest result to control group. In diabetic control group, macrophage levels were maintained high until the 14th day because the condition of the wound was still experiencing an inflammation.

The persistent inflammatory conditions inhibit wound healing. Hyperglycemia and oxidative stress also cause epigenetic changes which will affect the modulation and polarization of macrophages[28]. This polarization of deregulated macrophages is one of the main reasons of delayed wound healing. The antioxidant content of okra (*Abelmoschus esculentus*) pods has the ability to control hyperglycemia by reducing the activity of α-glucosidase and binding to free radicals[29].

The number of fibrocyte cells (Fig. 5) shows a significant difference between the treatment group and the diabetic control group. Whereas among the diabetic groups, there are no significant differences on the 3rd, 7th, and 14th days. The synthesis of fibroblasts, fibrocytes, and keratinocytes in diabetic mice is disturbed and decreased which causes a decrease in the secretion of growth factors and the synthesis of extracellular matrices such as collagen. Data of collagen density (Fig. 7) showed significant difference among the diabetic group, PAIO, and PAI, on the 7th and 14th days of treatment. Hyperglycemia decreases the synthesis of fibroblasts which follows by the decrease of synthesized collagen. Hyperglycemia also affects the healing process of wounds inflammation and epithelial cell migration by inhibiting the activity of both epithelial and immune cells[30].

CONCLUSION:

The administration of okra (*Abelmoschus esculentus*) pods extract combined with the alginate from *Sargassum ilicifolium* can increase the re-epithelialization rate of the wound area, the number of neutrophils, macrophages, fibroblasts, fibrocytes, as well as increase the collagen synthesis. The most optimal time of wound healing was on day 14 of the treatment. Therefore, it can be concluded that the administration of okra (*Abelmoschus esculentus*) pods extract-*Sargassum ilicifolium* alginate was able to improve open wounds healing in diabetic mice due to the role of antioxidant from quercetin and polysaccharides which acted as anti-hyperglycemic to anti-inflammatory properties.

ACKNOWLEDGEMENT:

Authors would like to thank the Dean of Faculty of Science and Technology and Head of Research and Community Services of Universitas Airlangga for the opportunity given to conduct this study funded by Mandat Research Grant, Universitas Airlangga 2019.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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Received on 01.09.2019 Modified on 21.10.2019
Accepted on 18.11.2019
Research J. Pharm. and Tech 2020; 13(6): 2764-2770.
DOI: 10.5958/0974-360X.2020.00491.6

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