

# Evaluation of the Combination of *Sargassum Duplicatum*, *Sargassum Illicifolium*, *Abelmoschus Esculentus*, and *Garcinia Mangostana* Extracts for Open Wound Healing in Diabetic Mice

Saikhu Akhmad Husen<sup>1,2\*</sup>, Muhammad Farraz Syadzha<sup>1</sup>, Muhamad Frendy Setyawan<sup>1</sup>, Pratiwi Pudjiastuti<sup>3</sup>, Arif Nur Muhammad Ansori<sup>4</sup>, Raden Joko Kuncoroningrat Susilo<sup>1</sup>, Suhailah Hayaza<sup>1</sup>, Dwi Winarni<sup>1</sup>, Win Darmanto<sup>1</sup>

<sup>1</sup>Department of Biology, Faculty of Science and Technology, Universitas Airlangga, 60115, Surabaya, Indonesia.

<sup>2</sup>Animal Histology Laboratory, Faculty of Science and Technology, Universitas Airlangga, 60115, Surabaya, Indonesia.

<sup>3</sup>Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, 60115, Surabaya, Indonesia.

<sup>4</sup>Doctoral Program in Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, 60115, Surabaya, Indonesia.

\*Corresponding Author: Saikhu Akhmad Husen

Department of Biology, Faculty of Science and Technology, Universitas Airlangga, 60115, Surabaya, Indonesia

E-mail: saikhu-a-h@fst.unair.ac.id

## ABSTRACT

**Introduction:** Diabetes mellitus (DM) is a chronic disease with diverse and complex metabolic disorders. DM can be identified with hyperglycemic conditions. In 2017, Indonesia ranked sixth in the world for the highest number of diabetic patients after China, India, United States, Brazil, and Mexico.

**Objective:** This study aims to determine the combined effect of alginate extract from *Sargassum duplicatum* and *Sargassum illicifolium* with okra (*Abelmoschus esculentus*) and mangosteen (*Garcinia mangostana*) pericarp extracts to ameliorate the wound width process, the number of neutrophil cells, macrophages, fibroblasts, fibrocytes, and collagen density in streptozotocin-induced diabetic mice.

**Methods:** This study used male mice (strain BALB/C, weighting 30±5 g each) which were grouped into four groups; normal control with water only; diabetic control group; combination treatment group with *S. duplicatum* extract, *A. esculentus* extract, and *G. mangostana* extract; and combination treatment group with *S. illicifolium* extract, *A. esculentus* extract, and *G. mangostana* extract. The diabetic control group and combination treatment groups were injected with multiple low doses of streptozotocin (STZ) at 30 mg/kg BW intraperitoneally. The combination extract dose given in treatment groups was 50 mg/kg BW. Measurement of fasting blood sugar levels was done before and after the STZ injection. The mice's skin was cut in the gluteal section as long as 1 cm, then treated topically in three different time periods namely 3, 7, and 14 days. Histological preparation and analysis were conducted on the 15<sup>th</sup> day.

**Results:** The administration of *A. esculentus* extract and *G. mangostana* extract combined with *S. duplicatum* and *S. illicifolium* increased wound healing indicator rate, decreased neutrophile cell count (NCC) and fibrocyte cell count (FCCC), and increased collagen synthesis as an extracellular matrix (ECM). The treatments could restore the open wound healing process of diabetic mice to their normal state. There were no significant differences in macrophage cell count (MCC) and fibroblast cell count (FBCC). The histopathology showed that the combination treatment groups also could accelerate the wound healing process in mice skin.

**Conclusion:** In summary, this study addressed that the administration of *A. esculentus* extract and *G. mangostana* extract combined with *S. duplicatum* and *S. illicifolium* extracts could ameliorate open wound healing in diabetic mice.

**Keywords:** *Sargassum*, *Abelmoschus esculentus*, *Garcinia mangostana*, open wound healing, diabetic mice

## Correspondence:

Saikhu Akhmad Husen

Department of Biology, Faculty of Science and Technology, Universitas Airlangga, 60115, Surabaya, Indonesia.

E-mail address: saikhu-a-h@fst.unair.ac.id

## INTRODUCTION

Diabetes mellitus (DM) is a chronic disease with diverse and complex metabolic disorders. DM can be identified with hyperglycemic conditions<sup>1</sup>. In 2017, Indonesia ranked sixth in the world for the highest number of diabetic patients after China, India, United States, Brazil, and Mexico. The number of Indonesia's diabetic patients in adulthood (18-99) is estimated at 10 million people with 7 million cases that have not been diagnosed and about 130 thousand cases of death from DM complications. It is estimated that one in 17 adults in Indonesia suffers from DM<sup>2</sup>. Hyperglycemia conditions can increase the production of reactive oxygen species

(ROS) which can cause oxidative stress<sup>3</sup>. Increased oxidative stress in diabetes, including through the formation of ROS, plays a major role in the pathogenesis of diabetes. Hyperglycemia in patients with DM can cause glucose auto-oxidation, activation of protein glycation, and activation of the polyol metabolic pathway which will accelerate the formation of ROS. Reactive oxygen species in the body will raise free radicals<sup>4</sup>. Increased levels of free radicals can damage proteins, lipids, and DNA, cause disruption in cellular function and even cause cell death<sup>5</sup>. Many complications can occur in people with DM such as ketoacidosis, retinopathy, nephropathy, neuropathy, cardiovascular disease, gastrointestinal disease, male

sexual dysfunction, and diabetic foot ulcer (DFU)<sup>6</sup>. A diabetic foot ulcer is an open wound that is difficult to cure due to impaired wound healing metabolism in people with DM<sup>7</sup>. The wound healing process is generally divided into stages of coagulation and hemostasis, inflammation, proliferative, and remodeling<sup>8</sup>. Many factors of metabolic disorders that interfere with the process of diabetic wound healing at each stage and one of them is an increase in ROS levels in diabetics. ROS can affect platelet aggregation and growth factor release, as well as induce an increase in MMP expression which will reduce ECM expression. Free radicals have the property to directly reduce the ECM component<sup>5</sup>. Excessive levels of ROS can be treated with antioxidants. Antioxidants are all substances that can delay or inhibit substrate oxidation. The physiological role of antioxidants is to prevent damage to cellular components that arise as a result of chemical reactions involving free radicals. In recent years, a large amount of evidence has developed supporting the key role of free radicals in many fundamental cellular reactions and suggests that oxidative stress may be important in the pathophysiology of common diseases including atherosclerosis, chronic kidney failure, and DM<sup>9</sup>. One class of antioxidants is phenolic molecules that are generally present in plants. *Abelmoschus esculentus* and *Garcinia mangostana* have phenolic compounds, quercetin, and alpha-mangostin, respectively, which have high antioxidant potential<sup>10,11</sup>. Algae from the genus *Sargassum* have phenolic compounds that act as antioxidants<sup>12</sup>. Phenolic compounds reduce or inhibit free radicals by transferring hydrogen atoms from their hydroxyl groups. The mechanism of the reaction of phenolic compounds with peroxy free radicals (ROO<sup>\*</sup>) involves the transfer of hydrogen cations from phenols to free radical molecules, forming the transition state of the H-O bond with one electron<sup>13</sup>. Therefore, this study aims to determine the effect of alginate combination from *S. duplicatum* or *S. illicifolium* with *A. esculentus* and *G. mangostana*. on open wound healing in diabetic mice by observing the width of the wound, the number of neutrophils, macrophages, fibroblasts, fibrocytes, and collagen density.

## MATERIALS AND METHODS

### Plant identification

Taxonomic identification of *S. duplicatum*, *S. illicifolium*, okra (*A. esculentus*) and mangosteen (*G. mangostana*) was carried out by the Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia.

### Extraction of *Sargassum duplicatum* and *Sargassum illicifolium*

Alginate extraction method referred to the Jakarta Center for Marine and Fisheries Product Processing and Biotechnology Research, Jakarta, Indonesia. Isolation of *S. illicifolium* and *S. duplicatum* alginate was carried out using 50 g of algae then adding 1.125 mL of 0.1% KOH. Then, around 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<sub>2</sub>CO<sub>3</sub> and 4% NaOCl, and the extract was blanched with a 2-propanol solution, and then sun-dried for 12 hours. The dried fiber was then crushed<sup>14</sup>.

### Extraction of *Abelmoschus esculentus*

All parts of the pods were used for extraction. *A. esculentus* pods were weighed 4 kg then blended and macerated with added 1 L of 96% ethanol until the solvent is clear. The solvent was evaporated with a rotary

vacuum evaporator at a temperature of around 50 °C, then the crude extract was weighed<sup>15</sup>.

### Extraction of *Garcinia mangostana*

*G. mangostana* pericarp was scraped and cut into small pieces and air-dried. Dry *G. mangostana* pericarp was weighed, then powdered using a blender and sieved. *G. mangostana* pericarp powder which had been dried was macerated with 96% ethanol and shook repeatedly until the solvent was clear. The solvent was evaporated with a rotary vacuum evaporator at a temperature of around 50°C until a crude wet extract was obtained. The wet extract was then freeze-dried using the freeze-drying tool and weighed<sup>16</sup>.

### Acute toxicity study

Mice which were treated with okra extract and *G. mangostana* pericarp extract had been tested for acute toxicity<sup>17</sup>. The dose of 50 mg/kg BB had been proven safe for experimental animals.

### Experimental animals and ethical approval

Male adult BALB/C strain mice were used aged 3-4 months old with body weight ranging from 30±5 g. The animals were obtained from the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. Mice were habituated to the Animal Laboratory environment, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia for two weeks. Bodyweight and blood glucose levels were measured pre and post administration of lard and STZ. Environmental conditions were controlled with a temperature of 25±5 °C, the humidity of 50±10% and 12 hours light/dark cycle. Mice were fed with standard pellet and drink (*ad libitum*). All treatment procedures had 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 with a completely randomized design at the Experimental Animal Laboratory and the Histology Laboratory of the Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia. This study was grouped into four groups; normal control; diabetic control group; combination treatment group with *S. duplicatum* extract, *A. esculentus* extract, and *G. mangostana* extract; combination treatment group with *S. illicifolium* extract, *A. esculentus* extract, and *G. mangostana* extract. Normal control group had only given with water. Therefore, diabetic control group and combination treatment groups were injected by STZ (30 mg/kg BW). The mice's skin was cut in the gluteal section as long as 1 cm, then treated topically in three different time periods on day 3, 7, and 14. Histological preparation and analysis were conducted on the 15<sup>th</sup> day. The dose value given to each combination treatment group was 50 mg/kg BW.

### Measurement of wound healing parameters

Neutrophils, macrophages, fibroblasts, and fibrocytes cells were counted utilizing a microscope equipped with a microscope grid whereas wound width and collagen density were analyzed using ImageJ software.

### Histological preparation

The mice's skin that had been cut were taken out and soaked in 10% formaldehyde for 48 hours. They were then cut into two, put into cassettes, and washed with running water for approximately 2 hours. Afterward, the skins were dehydrated using incrementally varying alcohol concentration and subsequently drenched in xylol

overnight. Then they were soaked in melted paraffin in preparation for the embedding process. After that, the skins were placed in a cubic mold in which the melted paraffin was then poured into, and they were left to harden. The hardened blocks of paraffin containing the skin were then sliced using a microtome and affixed to object glasses using Mayer's albumin. Following the affixion, the object glasses were heated in the oven at 50 °C and soaked in xylol followed by rehydrating using decreasingly varied alcohol concentration. They were then stained with hematoxylin and eosin (HE) for 10 minutes and rinsed with water. Stained histological preparation was then briefly put into 70% ethanol and HCl solution and then soaked in eosin stain for 5 minutes. Afterward, they were re-dehydrated with the increasing alcohol concentrations and put into xylol for 20 minutes. Lastly, cover glasses were mounted on top of the histological preparations with entellan.

**Data analysis**

Statistical analysis was carried out using SPSS (Statistical Package and Service Solutions, IBM) software version 17.0. The data obtained were analyzed with the Kolmogorov-Smirnov test and Levene's test. If the normality and homogeneity tests meet the requirements for parametric tests, the data will be further analyzed using One-Way Analysis of Variance ( $\alpha = 0.05$ ). If the data obtained is not homogeneous, it will be tested using the Brown-Forsythe test ( $\alpha = 0.05$ ) with the Games-Howell test to determine differences between the 2 groups.

**RESULTS**

**Effect of combination on wound healing parameters**

This study indicated that the combination treatment group was able to significantly reduce wound width, NCC, FCCC, and collagen density ( $p < 0.05$ ). However, no significance was generated for the MCC and FBCC parameters (Table 1)

**Table 1.** Effect of *A. esculentus* extract and *G. mangostana* extract combined with *S. illicifolium* or *S. duplicatum* extract on the parameters of open wound healing in diabetic mice.

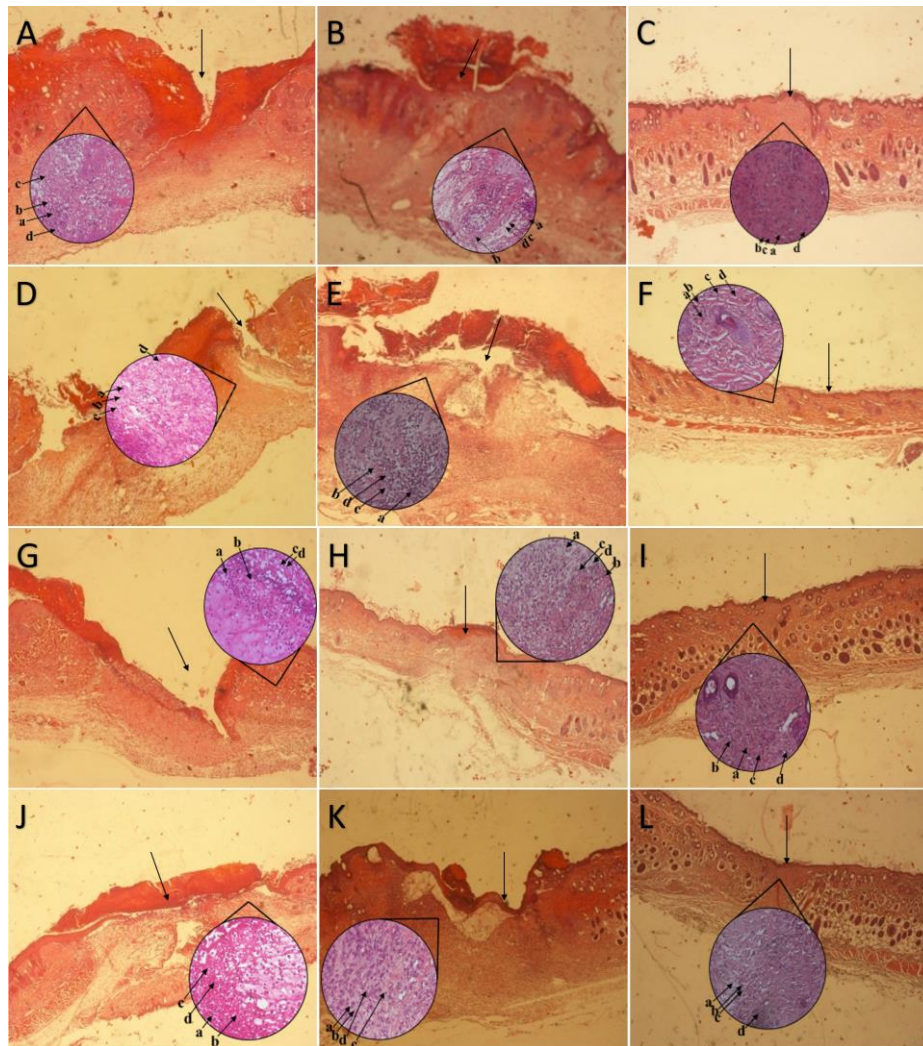
Groups	Wound Width (µm)	NCC	MCC	FBCC	FCCC	Collagen Density (%)
Control						
Day 3	2745.83±0.5	109±1	20.84±0.17	41.17±0.84	21±0.67	44.67±0.34
Day 7	1977.5±0.17	94±4	8.17±0.17	55±0.33	31±0.67	57.5±0.17
Day 14	100.5±0.17	20.17±0.17	3.67±0.34	33.5±0.17	18.5±0.17	96.84±0.17
Streptozotocin						
Day 3	2893.84±0.17*	188±1*	23.34±0.67	26±0.33	15.5±0.17*	40.67±0.34*
Day 7	2813±0.33*	161.5±0.5*	17.84±0.17	35.84±0.17*	20±0.33	54.17±0.17*
Day 14	203.165±0.17*	38.5±0.5*	7.67±0.34*	31±0.33	12.5±0.17	60.67±0.34*
<i>S. duplicatum</i> + <i>A. esculentus</i> + <i>G. mangostana</i>						
Day 3	2617.5±0.5**	76±1**	16±0.33	42±0.33	21.84±0.17	64.67±0.34**
Day 7	986.34±0.34**	69.5±0.5**	11±0.33+	55.17±0.17	35.5±0.17	77.5±0.17**
Day 14	15.84±0.17**	14.5±0.5+	4.5±0.17	35.5±0.17	16.5±0.17**	96.84±0.17+
<i>S. illicifolium</i> + <i>A. esculentus</i> + <i>G. mangostana</i>						
Day 3	1524.84±0.17**	76.5±0.5**	16±0.33	40.17±0.17	20.17±0.17*	65.67±0.34**
Day 7	770.5±0.17**	60.5±0.5+	3.5±0.17	57±0.67	37±0.67	80±0.33**
Day 14	9.165±0.17**	10.5±0.5+	0.84±0.17	36.5±0.17	17.17±0.17	97.17±0.5+

\*Note: Data express the mean±SD (n = 3). \*p <0.05 compared between the KN group and all groups. +p <0.05 compared between KD group and combination groups. NCC: Neutrophil cell count, MCC: Macrophage cell count, FBCC: Fibroblast cell count, FCCC: Fibrocyte cell count.

**Effect of combination on histology**

Based on the histopathological of wound healing parameters, significant differences between the normal, diabetic, and treatment groups were illustrated. In general, a combination treatment group could improve

diabetic wound healing similar to normal conditions. The two treatment groups gave the same effect with significant differences in wound width parameters (Figure 1).



**Figure 1. Histopathology of wound widths shown by black arrow lines.**

A: normal control on day 3; B: normal control on day 7; C: normal control on day 14; D: Diabetic control on day 3; E: Diabetic control on day 7; F: Diabetic control on day 14; G: combination treatment of *S. duplicatum* with *A. esculentus* extract and *G. mangostana* extract on day 3; H: combination treatment of *S. duplicatum* with *A. esculentus* extract and *G. mangostana* extract on day 7; I: combination treatment of *S. duplicatum* with *A. esculentus* extract and *G. mangostana* extract on day 14; J: combination treatment of *S. illicifolium* with *A. esculentus* extract and *G. mangostana* extract on day 3; K: combination treatment of *S. illicifolium* with *A. esculentus* and mangosteen pericarp extract on day 7; L: combination treatment of *S. illicifolium* alginate with *A. esculentus* extract and *G. mangostana* extract on day 14.

**DISCUSSION**

*A. esculentus* and *G. mangostana* are plants that are widely distributed in Indonesia. Moreover, *Sargassum* has high absorbance properties so that the wound exudate that is released will easily be absorbed by *Sargassum*. It also plays a role in optimizing the wound environment

and preventing the entry of bacteria from the surroundings<sup>18</sup>. *Sargassum* can form a gel that provides a moist environment which then accelerates granulation and re-epithelialization<sup>19</sup>. The second parameter observed was the number of inflammatory cells including neutrophils and macrophages. The number of neutrophil cells continues to increase 24-36 hours after injury, then decreases after several days<sup>8</sup>. Diabetic wounds are characterized by a prolonged inflammatory phase<sup>20</sup>. This was reflected by the high number of neutrophil cells and macrophage cells in the diabetic group compared to other groups. Hyperglycemic conditions cause impaired cytokine and growth factors expression that play a role in wound healing. This, coupled with an increase in ROS in hyperglycemic conditions exacerbate the situation<sup>5,20,22</sup>. Treatment with *S. duplicatum* and *S. illicifolium* extract along with *A. esculentus* extract and *G. mangostana* extract which has strong antioxidant potential can relieve ongoing inflammatory conditions. Fibroblast cells and fibrocytes produce cytokines and growth factors that play an important role in wound healing. In addition, these cells also produce collagen as

ECM which is crucial in wound healing as a medium for cell migration. In the condition of diabetes, it is found that both cells are few in number. Hyperglycemic conditions with reactive oxygen species produced inhibit the recruitment and performance of fibroblasts and fibrocytes<sup>20,21</sup>. Hindered fibroblasts and fibrocytes performance will affect the formation of ECM. This formation is also disrupted by ROS which increases MMP production which then degrades ECM<sup>5,22,23</sup>. Giving treatment with high antioxidant potential is able to restore the performance of these cells to a normal state, which in turn increases the collagen density back to its normal state.

## CONCLUSION

We conclude that the combination of *A. esculentus* extract and *G. mangostana* extract with *S. duplicatum* or *S. illicifolium* extract can increase the wound healing process, accelerate the prolonged inflammatory phase, and increase the number of fibroblast cells, fibrocytes and collagen density in the diabetic wound. It can be concluded that the administration of *A. esculentus* extract and *G. mangostana* extract with alginate of *S. duplicatum* or *S. illicifolium* can restore the healing process of open wounds in diabetic mice to normal conditions.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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