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Enhanced Fibrogenesis Activity in Diabetic Wound Using 20% *Anredera scandens* (L.) Moq. Leaves Extract Ointment in Sprague dawley Rats

(*Peningkatan Aktivitas Fibrogenesis pada Penyembuhan Luka Diabetik Menggunakan Salep Ekstrak Daun Anredera scandens (L.) Moq. 20% pada Tikus Sprague Dawley*)

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ABSTRACT

Background: In diabetic conditions, decreased glucose-6-phosphate dehydrogenase activity results in reduced nitric oxide synthesis. Reduced nitric oxide synthesis results in the production of ROS. This may impede the processes of angiogenesis and vasoconstriction and prolong inflammation. **Objective:** We were interested in investigating the mechanism by which 20% of *Anredera scandens* leaves extract ointment increases fibrogenesis activity because this condition can affect the development of damaged skin tissue. This study's design is a post-test-only laboratory experiment. **Material and methods:** The study employed male *Sprague dawley* strain rats that were induced with 40 mg/kgBW of STZ. The mice were divided into four groups: the negative group (which got diabetic wounds only), the positive group (which got Gentamicin ointment), and the test group (which got 20% extract ointment). **Results:** The skin tissue from each group was then examined histopathologically. After receiving 20% ointment of *Anredera scandens* leaves extract, the test group's mean fibrogenesis histopathology score on day 14 was significantly higher (P=0.022) at 3.75 than that of the negative group, which had a mean score of 2.75. Following that, the skin tissue of both the extract test group and the normal group was examined histopathologically. In comparison to the normal group mean score of 4.0 on day 14, the test group, which received 20% ointment of *Anredera scandens* leaves extract, had a non-significant (P=0.317) mean fibrogenesis histopathology score of 3.75. Subsequently, skin tissue was examined histopathologically for both the positive and normal groups. According to the results, on day 14, the positive group's average fibrogenesis histopathology score was 3.5, while the normal group's average score was 4.0. This difference was not statistically significant (P=0.127). **Conclusions:** Thus, it can be said that 20% *Anredera scandens* leaf extract ointment is comparable to gentamicin ointment in its capacity to promote fibrogenesis activity in diabetic wounds.

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INTRODUCTION

Higher-than-normal blood glucose levels are referred to as hyperglycemia. This ailment is among the signs of diabetes mellitus (DM). An unhealthy lifestyle that includes consuming sugar-rich meals and beverages on a daily basis is the cause of diabetes mellitus (DM). One of the most common effects of diabetes mellitus is diabetic wounds, sometimes referred to as diabetic neuropathic wounds, diabetic foot ulcers, or diabetic neuropathic wounds. Diabetic wounds are common in people with diabetes and impact the peripheral and autonomic nerves. Tissue necrosis, or dead tissue, brought on by an embolism in a major artery in a particular part of the body that stops the blood flow is what is known as diabetic wounds with gangrene. can be caused by long-term inflammatory conditions, occupational accidents, degenerative conditions (arterioschlorosis), or metabolic diseases (diabetes mellitus) (Lipsky et al., 2012).

Nitric oxide production is decreased in diabetic conditions due to decreased glucose-6-phosphate dehydrogenase activity. Reduced nitrooxide synthesis results in the production of reactive oxygen species (ROS). According to Patel et al. (2019), this may exacerbate inflammation and slow down the processes of angiogenesis and vasoconstriction. Cytokines are essential for regulating inflammation during the healing process of wounds. Among the cytokines are interferon (INF), interleukin (IL), and tumour necrosis factor (TNF). Interleukins come in different forms, such as IL-1α and β, IL-2, IL-6, IL-8, IL-4, and IL-10. Tissue macrophages and blood monocytes both generate the essential inflammatory molecule IL-1β. This cytokine is triggered by caspase-1 cleavage in secretory lysosomes, which occurs subsequent to caspase-1 activation by the NALP3 inflammasome. Apart from initiating inflammation, IL-1β also stimulates inflammation by increasing the mobilization of leukocytes derived from bone marrow and acute phase proteins secreted by the liver. The impact of IL-1β levels on the rate of wound healing is widely acknowledged (DeClue and Shornick, 2015). It has been demonstrated that lowering IL-1β levels in diabetic rats speeds up the pro-inflammatory to anti-inflammatory or reparative macrophage phenotype transition, which in turn promotes the synthesis of goth factor and speeds up the healing of wounds (Mirza et al., 2013). As a result, it might trigger inflammation sooner, which might trigger the proliferative phase that will see an increase in fibrogenesis activity.

Anredera scandens (L.) Moq. has long been used as a wound remedy by Indonesians (Manoi, 2009). Previous reports from scientific studies on the wound-healing properties of *Anredera scandens* (L.) Moq. According to histopathology, 70% of the *Anredera scandens* (L.) Moq. extract has anti-burn properties (Karismawan, 2013). Extract 95% of *Anredera scandens* (L.) Moq. It also has an anti-ulcer effect for the healing of internal wounds, according to research on Sprague Dawley rats (Samirana et al., 2015). Furthermore, studies using concentrations of 10% and 30%, which are equivalent to chloramphenicol, have demonstrated the ability of Anredera cordifolia (tenore) steen, another variety of the Binahong plant, to treat diabetic wounds (Kintoko & Desmayanti, 2016). But the nature of the mechanism of diabetic wound healing is not made clear in detail.

Triterpenoids, saponins, tannins, and flavonoids are a few examples of secondary metabolite compounds found in the leaves of *Anredera scandens* (L.) Moq. (Samirana et al., 2015). The anti-wound properties of the tannin group of compounds are widely recognized. According to Shenoy et al. (2012), their mode of action entails binding free radicals, encouraging wound contraction, and promoting capillary and fibroblast growth. Apart from that, one of the secondary metabolites of *Anredera scandens* (L.) Moq has been found to be a flavonoid class compound with the chemical formula 4',7 dihydroxy 3-O-R flavonol (Yadnya Putra et al., 2019). It is commonly known that the mechanisms of action of flavonoids and NSAIDs involve the suppression of pro-inflammatory mediator gene expression or activity. Furthermore, flavonoids have the capacity to stop the release of cytokines (Comalada et al, 2006). Consequently, the flavonoid compounds of *Anredera scandens* (L.) Moq. Owing to its anti-inflammatory characteristics and ability to prevent the production of IL-1β, it is thought to have a secondary impact on the process of wound healing. The possibility that *Anredera scandens* (L.) Moq. can heal diabetic wounds is therefore thought to exist. This could serve as a foundation for the discovery of alternative diabetic wound-healing therapies, which would also allow for the potential reuse of natural ingredients and hopefully lessen human reliance on synthetic drugs.

MATERIAL AND METHODS

Materials

P20% ointment binahong leaf extract; Gentamicin Ointment; streptozotocin (STZ); Masson trichrome coloring reagent; BNF (Buffer Neutral Formaline); ketamine; Surgical tools; microscope. 20% ethanol extract from *Anredera scandens* (L.) Moq., 0.25% methyl paraben, 0.15% propyl paraben, 1% sodium lauryl sulfate, 12% propylene glycol, 25% stearyl alcohol, 25% white petrolatum, and distilled water until 100% are used to make the ointment. Stearyl alcohol and white petrolatum are melted in a water bath at 65° to 70° C to start the manufacturing process. then raising the mixture temperature (Mixture A) to approximately 75° C. In distilled water, combine additional ingredients, such as a thick extract of *Anredera scandens* (L.) Moq leaves, and bring to a temperature of 75°C (Mixture B). Slowly stir in Mixture B and add it to Mixture A. Take the mixture off of the heat and stir until it thickens.

In vivo test

After one week of adaptation, twenty-four male white Sprague Dawley rats (weighing 200–250 grams and aged 10–12 weeks) were split into four test groups, with four rats (Sample) in each small group.

Methods

In order to guarantee that study adheres to research ethical guidelines, authorization must be obtained prior to beginning any research. One of the permits for doing research is the completion of an ethical clearance study prior to any research being conducted. The udayana university faculty of medicine research ethics commission has granted ethical clearance through a letter with the number 674/UN14.2.2.VII.14/LT/2021.

Diabetes induction process

A single intramuscular injection of freshly prepared STZ (40 mg/kg) in cold 0.1M sodium citrate buffer (pH 4.5) will be given to twenty animals each from the test, positive, and negative groups. The animals will then fast for about sixteen hours, during which they will only be given water when they need it. Three days after induction, a drop of blood (±1.5 µl) was taken from each mouse's tail vein using a lancet. Blood tests with a glucometer are performed to identify the onset of hyperglycemia. If the rats in this study had a fasting blood glucose level of 126 mg/dL or more, they were considered diabetics and used in the experiments that followed (Tan et al. 2019).

Wound creation

The animals were given 10 mg/kg of xylazine and 90 mg/kg of ketamine intramuscular for anesthesia after it was confirmed that they had diabetes. The animals' back fur was cleaned with 70% ethanol and shaved with electric clippers before the wound was created. Using a biopsy punch, a full thickness excision wound with dimensions of 6 mm in diameter and 2 mm in depth was created. The wound was left open for the entire day. Rats with open wounds were kept in cages specifically made to accommodate separating rats.

Histopathology sample preparation

100 mg/kg BW of sodium pentobarbital was injected intraperitoneally to put the animals to sleep. Following animal sacrifice, the test animal's wound tissue was cut into pieces measuring 0.5 x 0.5 cm and fixed for 48 hours using a 10% BNF (Buffer Neutral Formaline) solution. Next, ethanol concentrations of 50%, 90%, and pure ethanol were used for 30 minutes each to dehydrate the tissue, and finally, the impregnation clearing procedure and block-making were carried out. After slicing the blocks into ribbon-shaped pieces using a microtome to cut them to a thickness of 5 µm, the water was heated to 46°C. After that, the resultant sections were taken out and set on glass surfaces to dry for the night.

Histopathological analysis

The skin tissue was then examined under a 400-times magnification light microscope. Histopathology was examined from five different perspectives. The results of each field of view are recorded on camera, and the fibroblast cell counts of the test group and the positive control are compared to assess or grade the outcomes. The percentage value is then assigned a score between 0 and 4.

RESULTS AND DISCUSSION

There are several different secondary metabolites in binahong leaves (*Anredera scandens* (L.) Moq.). Extract *Anredera scandens* (L.) Moq to learn this. This secondary metabolite has the ability to heal wounds, especially diabetic wounds. Based on the criterion of increasing fibrogenesis activity, it is known from this study that binahong leaf extract ointment (*Anredera scandens* (L.) Moq.) works well to treat diabetic wounds. Comparing the treatment groups demonstrates this; the extract test group displayed a significant histopathological picture based on scoring values validated by statistical analysis. The test animals were first given STZ (streptozotocin) to induce diabetes before a 1 cm incision was made. Blood samples will be taken on the 7th and 14th days prior to sacrifice in order to determine the test animals' blood sugar levels. The following table 1 displays the test group's average blood sugar levels.

Table 1. Average blood sugar levels

Group	Test	Average blood sugar levels (mg/dl)	SD
K1	Normal	96.33	±7.90
K2a	Gentamicin day 7	184.84	±9.68
K2b	Gentamicin day 14	257.18	±30.73
K3a	Diabetic wound day 7	173.26	±7.21
K3b	Diabetic wound day 14	265.25	±67.74
K4a	Extract 20% day 7	169.77	±24.53
K4b	Extract 20% day 14	225.49	±66.62

The World Health Organization states that blood sugar levels during a fast should be between 100 and 126 mg/dl. Diabetes is defined as having blood sugar levels below this threshold. It can be concluded that the test animals in this study were already diabetics based on Table 1's data that each treatment group's blood sugar levels exceeded the diabetes blood sugar level limit. As a result, we can continue to observe diabetic wound healing based on increased fibrogenesis activity.

Based on the average scoring value as indicated in Figure 1, the positive group was known to have increased fibrogenesis activity on day 7, but it did not demonstrate a significant difference until day 14. It is visually evident from Figure 1a that there are still a lot of fibroblast cells. which demonstrates that the absence of collagen fibres is the reason the wound is still not healing fully. The diabetic wound then closed on the fourteenth day (Figure 1b), indicating that the fibrogenesis process had started. At this point, it was observed that the fibroblast cells had started to shrink and transform into collagen fibres.

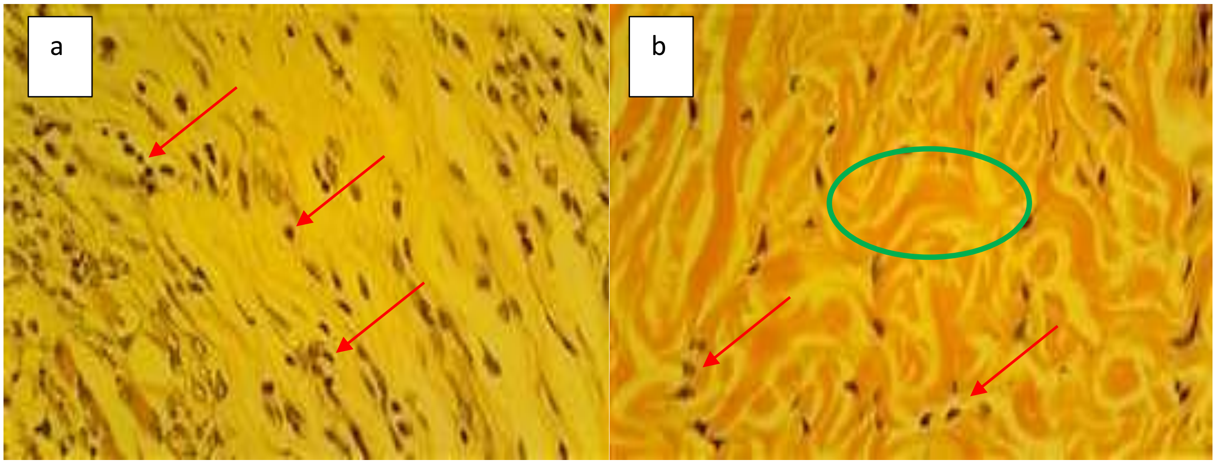

Figure 1. Positive group at day 7 (a) and day 14 (b) (red arrow: fibroblast cell; green circle: collagen tissue)

This is believed to happen because bacteria have the ability to affect how quickly diabetic wounds heal. Topical gentamicin is administered as a positive control because wound healing will proceed more quickly if bacterial infection can be avoided. Bacterial colonisation and proliferation in diabetic foot ulcers are thought to significantly impede wound healing, according to Smith et al. (2016). Diabetes wounds become more chronic as a result of bacterial colonies forming biofilms (Noor et al., 2015). In biofilms, bacterial colonies secrete a matrix called a glycocalyx that protects them from the host immune system and prevents them from being eliminated.

After the fourteenth day, out of all the groups depicted in Figure 2, the negative group had the lowest average scoring value. As seen visually in Figure 2a, there are still a lot of fibroblast cells, indicating that the wound has not yet healed completely and that collagen fibres have not yet formed. Then, on day 14 (Figure 2b), it was observed that the fibroblast cells were still there, albeit in greater quantities. This resulted in the diabetic wound's inflammation lasting longer, which impeded the continuous process of fibrogenesis.

This confirms the theory that the high concentration of sugar in diabetic wounds will lower the activity of glucose-6-phosphate dehydrogenase, which in turn will lower the synthesis of nitric oxide if the wounds are not treated right away. Reactive oxygen species (ROS) are produced when nitrooxide synthesis is reduced. This can prolong inflammation and slow down the angiogenesis and vasoconstriction processes (Patel et al. 2019). A statistically significant difference ($p<0.05$) was observed between the negative, normal, and extract test groups, as well as between the positive and negative groups. This difference may be attributed to the death of pancreatic beta cells, which exacerbates the inflammatory state in diabetic wounds.

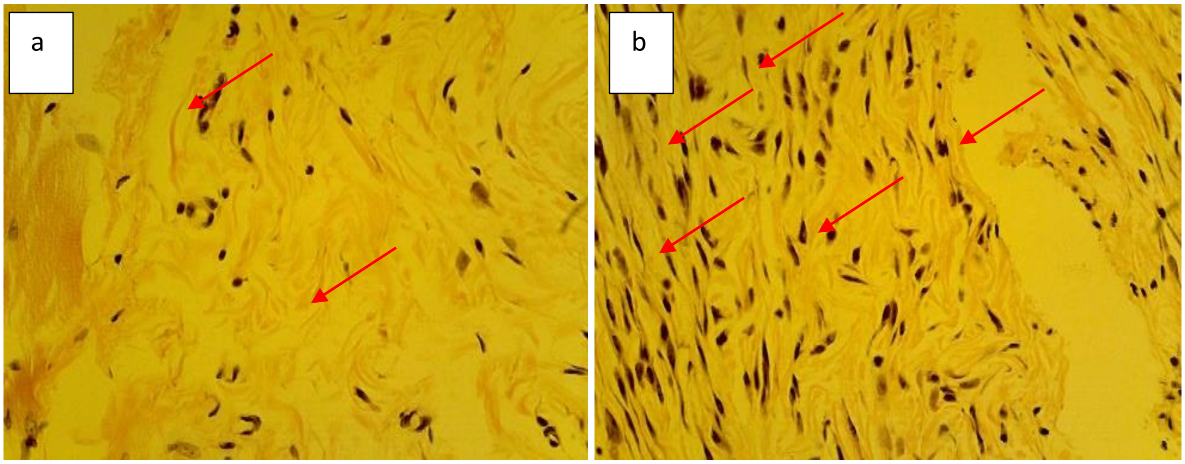

Figure 2. Negative group at Day 7 (a) and day 14 (b) (red arrow: fibroblast cell)

When compared to the positive control depicted in figure 3, the histopathological picture of the 20% extract test group demonstrates an increase in fibrogenesis activity from day 7 to day 14, which has a statistically significant difference ($p<0.05$). Figure 3a illustrates this. The diabetic wound has begun to heal on the seventh day, although there are still fibroblast cells present. Collagen fibres have begun to form, but they are still not perfect. In the meantime, histopathology, where the collagen fibres have fully started to form, confirms that the wound has closed completely in Figure 3b. The secondary metabolite content of the leaf extract of *Anredera scandens* (L.) Moq is thought to be the cause of this. *Anredera scandens* (L.) Moq. leaves are known to contain flavonoids, saponins, triterpenoids, and tannins, according to phytochemical tests. Additionally, a number of studies have demonstrated the capacity of tannins, flavonoids, saponins, and triterpenoids to promote wound healing.

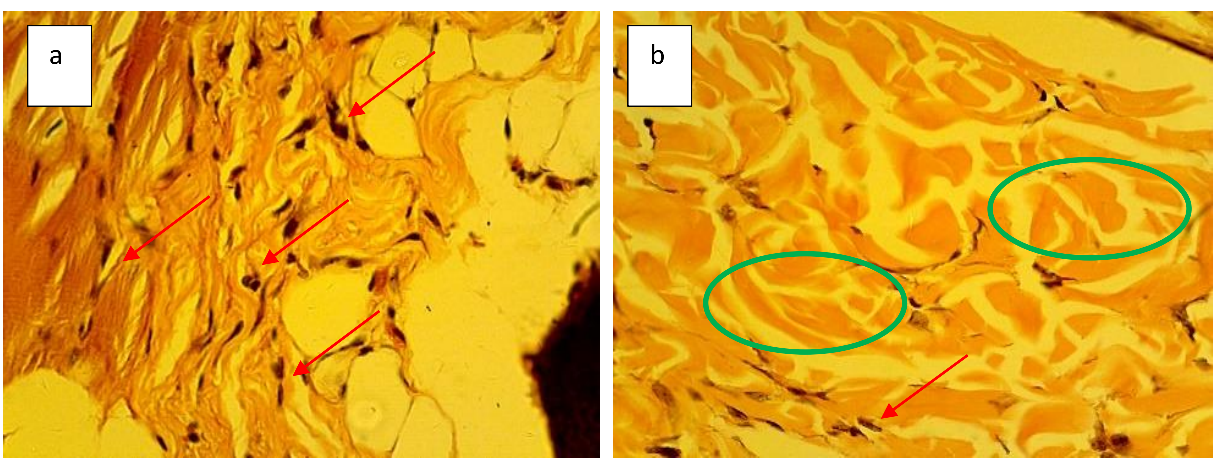

Figure 3. Extract 20% at Day 7 (a) and day 14 (b) (red arrow: fibroblast cell; green circle: collagen tissue)

Following the display of the histopathological image, a scoring evaluation based on the developed collagen fibers will be conducted. The first score (Small connective tissue, sparse or not compact and unevenly distributed) is the criterion used for scoring. The wound is still open; score 2: There is little connective tissue, but it has gathered in multiple areas. Open or closed wounds receive a score of 3 (tight, dense connective tissue). score 4 (Dense and compact connective tissue); the wound is closed, but a cavity remains. No cavities remain, and the wound has closed.) Table 2 displays the outcomes. Table 2 displays the average value of the test group on days 7 and 14, as well as the scoring value for each sample. On the fourteenth day, the negative group displayed the lowest scoring value, while the 20% extract group demonstrated the best score since it was the closest to the value of the normal group.

Table 2. observation of scoring between treatment groups

Group Observation day 7				
Rats	Normal	Positive	Negative	Extract 20%
1	4	3	3	3
2	4	2	2	3
3	4	2	2	3
4	4	2	1	2
mean	4	2.25	2	2.75
SD	0.000	0.50	0.82	0.50
Group Observation day 14				
Rats	Normal	Positive	Negative	Extract 20%
1	4	4	3	4
2	4	3	3	4
3	4	3	2	4
4	4	4	3	3
mean	4	3.5	2.75	3.75
SD	0.00	0.57	0.50	0.50

Anredera scandens (L.) Moq leaves, 20% extract, have been shown in studies to have wound-healing properties comparable to or better than those of gentamicin, as a positive control, for diabetic wounds. Conversely, the negative group's fibrogenesis developed at the slowest rate. Numerous things, such as the development of bacterial colonies or an extended inflammatory process, can have an impact on this. To determine statistical differences in the increase in fibrogenesis activity in each group, further tests were carried out using the Mann-Withney test. The data is displayed in the following table 3.

Table 3. Comparison Data of Histoptological Scoring Between Groups Using the Mann-Whitney Test

Day 7			
Group		P Value	Explanation
Normal	Positive	0.011	Significantly different
	Negative	0.013	Significantly different
	Extract 20%	0.011	Significantly different
Negative	Positive	0.617	Not Significantly different
	Extract 20%	0.155	Not Significantly different
Day 14			
Group		P Value	Explanation
Normal	Positive	0.127	Not Significantly different
	Negative	0.011	Significantly different
	Extract 20%	0.317	Not Significantly different
Negative	Positive	0.032	Significantly different
	Extract 20%	0.022	Significantly different

As can be seen in Table 2, on day seven there was a significant difference (P<0.05) between the normal group and all test groups, whereas the negative group and the extract test showed a non-significant difference (P>0.05). Subsequently, it was observed that, on the fourteenth day, the negative group had a significant difference when compared to all groups, while the normal group had no significant differences with either the positive or extract test groups. This demonstrates that 20% extract had the same capacity to enhance fibrogenesis activity with gentamicin on the fourteenth day of *Anredera scandens* (L.) Moq leaves, approaching the skin in the normal group. The contents of *Anredera scandens* (L.) Moq's different secondary metabolites may be the cause of this. Tannin, triterpenoid, flavonoid, and saponin compounds are found in *Anredera scandens* (L.) Moq's secondary metabolites. Leaves of *Anredera scandens* (L.) Moq. contain flavonoid compounds. Yadnya Putra et al. (2019) reported that the chemical formula of 4',7 dihydroxy 3-O-R flavonol. Several studies show that flavonoids can play a role in diabetes mellitus (Testa et. al. 2016). A screening test for phytochemicals was used to get the results. The extracted extract tested positive for the presence of secondary metabolites in the form of tannins, flavonoids, tritepenoids, and saponins, according to the results of the phytochemical screening. Table 4 illustrates this.

Table 4. Phytochemical Test Results of Binahong Leaf Extract (*Anredera scandens* (L.) Moq.)

No.	Metabolite	References	Result	Conclusion
1	Tanin	develops into a blackish green or dark blue color[1]	A greenish-black shade appears.	Positive Tanin
2	Flavonoid	Under UV 366 nm, the solution fluoresces intensely yellow[3]	Strong yellow fluorescent color	Positive Flavonoid
3	Triterpenoid	Violet or brownish ring[2]	A ring that is brownish appears.	Positive Triterpenoid
4	Saponin	A persistent ± 10-minute-long foam that reaches heights of 1–10 cm remains after a single drop of HCl 2N3 is added.[3]	After 10 minutes, foam as high as 2 cm developed and persisted after one drop of 2N HCl was added.	Positive Saponin

([1]Robinson, 1991;[2]Ciulei, 1984;[3]Depkes RI, 2020)

Flavonoids

It is well known that flavonoids affect oxidative stress, insulin resistance, and stress sensitivity signalling pathways, lipid and carbohydrate metabolism, hyperglycemia, and inflammatory processes (Choi and Kim 2009). The inflammatory cytokines IL-1β, IL-6, and TNF-α have been shown to be effectively reduced by the flavonoids morin, hesperidin, routine, and chrysin in diabetic animals, leading to a significant improvement in hyperglycemia, glucose intolerance, and insulin resistance (Abuohashish et al. 2013; Niture et al., 2014). Diabetes mellitus can lead to secondary damage to the kidneys, heart, nerves, eyes, and kidneys. Flavonoids regulate secondary damage to various peripheral organs in addition to helping restore glucose homoeostasis, which is necessary to treat debilitating diabetic conditions (Visnagri et al. 2014). As a result, this speeds up the inflammatory process, which has an indirect impact on fibrogenesis activity, which happens during the proliferation phase that follows inflammation.

Saponins and triterpenoids

It is believed that the binahong plant's saponin compound, which also plays a part in Centella asiatica's saponin, has an antibacterial function in reducing the severity of diabetic wounds. (2016) Mahmood et al. In addition, a number of sources claim that compounds made of saponins have anti-inflammatory qualities. A class of naturally occurring substances known as saponins is made up of glycosides, which are non-sugar substances, and sugar chains, which are sugar substances, normally joined by glycosidic bonds. Steroids (C-27) and triterpenoids (C-30) are examples of saponins. Triterpenoids and saponins, at various concentrations, have been shown to exhibit specific antibacterial activity against biofilm bacteria when linked to one or more molecules of glycone (Kacar et al., 2018). Because saponins and triterpenoids have antibacterial properties, they can reduce inflammation and speed up the proliferation stage, which is when fibrogenesis activity occurs.

Tannin

The antibacterial properties of the tannin compounds in the binahong plant aid in the healing of diabetic wounds by reducing their severity. Zhang et al. (2020) state that the tannin group's secondary metabolites are fairly effective at preventing the growth of biofilms from bacterial colonies of S. aureus, P. aeruginosa, and E. coli. Tannins are described as "C- and O-glycosidic derivatives of gallic acid (3,4,5-trihydroxybenzoic acid)" in textbooks on organic chemistry. Condensed tannins based on flavan-3-ol (catechin) units are not included in this definition, which also only covers a portion of tannins. Tannic acid, also known as tannic acid derivative (Khanbabaee and van Ree 2001), is one derivative of the tannin compound. This is consistent with saponins' and triterpenoids' capacity to act as antibacterials and reduce inflammatory conditions. However, tannin is known to have the ability to stop bacteria from growing, which is common in diabetic wounds.

CONCLUSION

Research indicates that a 20% extract of binahong leaves (*Anredera scandens* (L.) Moq.) can speed up the proliferation phase, which in turn can speed up fibrogenesis activity by lessening the impact of inflammation on diabetic wounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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