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Wound healing potential of *Tinospora Crispa* (Willd.) Miers [Menispermaceae] stem on diabetic mice

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Abstract
Tinospora crispa of the family Menispermaceae has been traditionally used in the Philippines to treat a variety of illnesses, including diabetes milletus and its accompanying complications. This study aimed to compare the effect of *Tinospora crispa* on healing of diabetic wound among albino mice when given intraperitoneally and when administered along with an ointment topically applied to the wound. Results showed a significant reduction in blood glucose level among mice given the plant extract. However, it may be suggestive that the additional ointment had not contributed significantly to wound healing as the wound healing time and percent wound contraction between the two groups are not significantly different.

Keywords: *Tinospora crispa*, Diabetes milletus, Menispermaceae, diabetic wound, healing

1. Introduction
Tinospora crispa of the family Menispermaceae is a climbing shrub which can grow up to 15 meters long. It is widely distributed in Southeast Asia and China and has been used by households as a cheap alternative medicine for various ailments. Traditional uses include treatment for hypertension, wounds, intestinal worms and skin infections [1], stimulation of appetite and protection from mosquito bites [2]. Scientific researchers have elucidated on its many biological activities, especially the stem, such as the antioxidant activity of the methanolic extract [3], immunomodulatory effect of isolated fraction from the stem [4], anti-inflammatory activities of the aqueous extract [5], and its analgesic and antimicrobial activities [6]. The roots also exhibit antimicrobial effects [7].
Phytochemical analysis of the plant revealed the presence of alkaloids [8, 9], phenols and flavonoids [10], and a variety of active compounds and secondary metabolites [11].
Tinospora crispa, locally known as "makabuhay" or "papaitan" in the Philippines, is also used in rural areas to treat diabetes mellitus [12]. A study by Lokman and colleagues [13] have shown the antidiabetic effect of oral borapetol B, a compound isolated from the plant and stimulates the release of insulin, a possible major treatment route in the treatment of type II diabetes mellitus. *Tinospora cordifolia*, a member of the same plant family, share a similar antidiabetic characteristic [14].
The total number of people with diabetes is projected to rise to 366 million in 2030[15]. Diabetes mellitus type II is a chronic metabolic disease that is characterized by abnormal level of blood sugar in the body because of the pancreas' inability to produce or too little production of insulin. Although it is now confirmed that the disease may be a product of many genetic factors [16] and that individuals may be genetically predisposed to acquiring the disease [17], a healthy and active lifestyle may still decrease the risk of susceptibility [18].
For people, however, who acquired the disease in their lifetime, multiple complications may arise including the inability for diabetic wounds to heal properly. Diabetic foot ulcer is a leading cause of amputations and affects 15% of people with diabetes [19]. Although topical treatment is considered an important aspect of wound care, Higgins and Ashry [20] emphasized that this should be secondary to surgical and systemic care. White and McIntosh [21] propose that some hard-to-heal ulcers may not be treated by application of topical treatments alone.
In this context, the researchers investigated the combined effects of *Tinospora crispa* as a systemic hypoglycemic agent to reduce blood sugar and as topical ointment on diabetic wound as compared to those only administered with extracts.

2. Materials and Methods

2.1. Preparation of Extract

Fresh stems of *Tinospora crispa* were obtained from a local source, air dried for 24-48 hours, and cut into small pieces. These were soaked in ethanol for 48 hours in a 1:1 w/v ratio. These were strained afterwards. Simple distillation was done to separate the ethanol from the extracts. Extract was stored in the refrigerator at 4C before use.

2.2. Preparation of Topical Ointment

Preparation of *Tinospora crispa* ointment was done using a common household procedure in the Philippines. Fresh stem weighing 100g were cut into small pieces and slowly fried until crispy in 200 ml of vegetable oil. Stems were removed while the oil was mixed with two wax candles (Esperma 5) sliced into small cubes. All debris were strained. The wax was thoroughly mixed and allowed to melt and settle.

2.3. Experimental Animal Groups

Albino mice 8-10 weeks old and about 25-30 grams were used in the study. The animals were acclimatized for seven days before proceeding with the experiment. All methods employed were presented to a research committee tasked to appraise the procedure and ensure that it complied with existing protocol. Additionally, researchers strictly adhered to the Code of Practice for the care and use of laboratory animals in the Philippines prepared by the Philippine Association for Laboratory Animal Science (PALAS).

Mice were grouped into the following treatments with five replicates each:

Group A – did not receive any treatment and ointment
Group B– given extract IP once a day and ointment twice a day for fourteen days
Group C – given extract only once a day for fourteen days
Group D – positive control (Bactroban) for fourteen days

2.4. Induction of Diabetes

Diabetes was induced among mice by intraperitoneal injection of 150 mg/kg body weight of alloxan monohydrate, dissolved in 0.9% saline [22]. Mice with blood glucose exceeding 250 mg/dL were considered diabetic and included in the study. Glucose was monitored before treatment, one hour after first treatment and fourteen days after treatment.

2.5. Incision of Wound

Diabetic mice were subjected to wound incision in the dorsal thoracic shaved skin after topical sterilization with alcohol. Wound was 10 mm in length and were left open. Mice were anaesthetized prior to creation of wound by intramuscular injection of Zoletil anesthesia (0.1 mL/kg body weight).

2.6. Evaluation of Wound

Gross examination of the wound was done daily to check on color, presence of exudates, swelling and consistency of tissues surrounding the wound [23]. Wound size was computed based on the following formula:

$$\% \text{ wound closure} = \frac{\text{Initial area of wound} - \text{final area of wound}}{\text{Initial area of wound}} \times 100$$

Although experiment ended on day 14, mice were still observed to note the number of days by which the wound have completely healed.

2.7. Histological Examination

Representative skin tissue samples were collected after sacrificing the mice for histological examination following methods by Yaman and colleagues [24]. Tissues were fixed in 10% neutral – buffered formalin solution and brought to the Bicol Regional Teaching and Training Hospital (BRTTH) Pathology section for preparation of slides. Prepared slides stained with Hematoxylin and Eosin were observed under light microscope.

2.8. Statistical Analysis

Numerical data were analyzed using Analysis of Variance (ANOVA) at 5% level of significance and Duncan Multiple Range Test (DMRT) as post-hoc test. Statistical analysis was carried out using SPSS version 22.

3. Results

3.1. Blood glucose level

Because diabetes is a systemic disease, the blood sugar among the experimental animals were checked after one hour of treatment and every day for fourteen days. Table 1 summarized the start and end of the blood sugar monitoring. Untreated mice never reduced its blood glucose level while the positive control recovered slightly, almost negligible, after fourteen days. *Tinospora crispa* extracts, given intraperitoneally, have significantly decreased the blood glucose level by almost fifty percent after fourteen days.

Table 1: Change (in percent) in glucose level of mice after one hour of treatment and fourteen days

Group	After 1 hour after treatment*	Day 14*
A (untreated)	-4.18^{c}	-7.25^{c}
B (extract and ointment)	27.74^{a}	55.90^{a}
C (extract only)	15.59^{b}	48.88^{a}
D (control – Bactroban)	-2.32^{c}	2.68^{b}

** – significantly different at p<0.05*
Values followed by similar letters are statistically comparable

3.2. Wound Contraction

Gross examination of the wound size suggested a slow recovery for untreated hyperglycemic mice. Wound have not completely healed on day fourteen, compared to a completely closed wound for those given the *Tinospora crispa* extracts. Comparing groups B and C, topical ointment seem not to hasten the wound healing. It may be suggestive that wound healing is primarily brought by a systemic reduction of blood sugar that improves blood circulation to the affected area, and may be secondarily because of the ointment to reduce risk of infection.

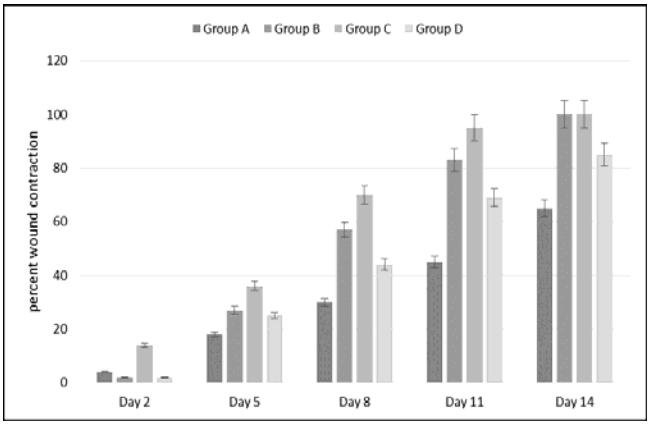

Fig 1: Effect of treatments to diabetic wounds. Values are mean ±SEM.

3.3. *Epithelialization Time*

It took less time for wounds to heal when mice were given *Tinospora crispa* extracts compared to the untreated and positive control group. Average epithelialization time for groups B and C was twelve days.

Table 2: Average wound healing time (in days) of wounds in diabetic mice

Group	Average Epithelialization Time*
Group A (untreated)	17.60±*0.5*[a]
Group B (extract and ointment)	12.80±*0.5*[c]
Group C (extract only)	11.20±*0.5*[c]
Group D (control – Bactroban)	15.60±*0.5*[b]

** – significantly different at p<0.05*
Values are means ± standard deviation
Values followed by similar letters are statistically comparable

3.4. *Histological Examination of Wounds*

Tissue samples of the untreated mice showed mild chronic inflammation in the dermis, moderate epidermal necrosis but with negative ulceration and granulation tissue. A similar histological characteristic was seen in the sample from those given the extracts and ointment. On the other hand, those given the extract alone showed no chronic inflammation in the dermis, with moderate epidermal necrosis and has no negative ulceration and granulation tissue.

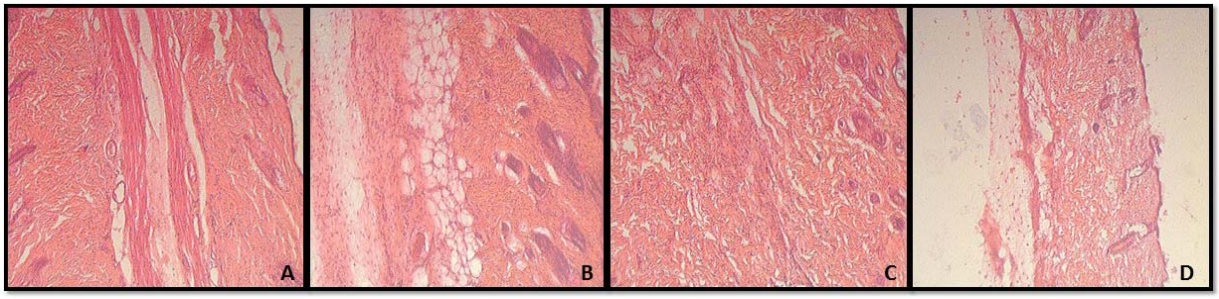

Figure 2. Excised wounds from diabetic mice given no extract and ointment – negative control (A), administered with *Tinospora crispa* extract and *Tinospora crispa* ointment (B), with *Tinospora crispa* extract only (C), and with *Bactroban* (positive control) ointment (D). (H&E, 400x)

4. Discussion

Wound healing among diabetic patients is physiologically impaired. Peripheral neuropathy and peripheral arterial diseases occur with diabetes resulting to narrower blood vessels that reduce circulation to areas that need healing. Reducing blood supply means less oxygen and nutrients to affected body parts. Ischemia, neuropathy and microbial infection [25, 26] are considered the three components that lead to diabetic complications such as poor and delayed wound healing. Most often, extremities are the ones likely to develop such complications. Diabetic foot ulcer (DFU) is the most common.

Blood sugar control, wound debridement and advanced dressing are among the most important ways to regulate diabetic wounds [27, 28]. Regulating blood glucose level is the most important metabolic factor that enhances wound healing. Elevated glucose level leads to excessive neutrophil infiltration which contributes to impaired healing process [29].

Topical treatments applied on diabetic wounds work to provide antimicrobial activity and prevent infection. Wound dressings and ointments should provide a moist environment while controlling microbial growth [30, 31]. However, it may not always work without balancing wound management with blood glucose reduction and optimizing other modalities of treatment. Among patients with diabetic wounds, especially diabetic foot ulcers, pharmacological interventions should be optimized in addition to other forms of treatment mechanisms [32].

Many plant materials have been tested on both their ability to reduce blood sugar and their wound healing property. Some examples are the bark of *Polyalthia longifolia* [33], *Allium cepa* [34], *Ficus mollis* [35] and *Ocimum sanctum* [36] among others. The use of plants in tropical countries is a common practice for a variety of reasons: availability, relative affordability and ease in handling and preparation. For a systemic disease such as diabetes, plants provide alternative relief to its complications. *Tinospora crispa* may be a potential source of new biochemical compounds to advance researches in diabetes care and management.

5. Conclusion

From the results of the study, *Tinospora crispa* stem extracts showed potentials to be harnessed as a remedy for diabetic wounds, primarily in reducing blood glucose level and second, in possibly preventing microbial infection in affected area.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

6. Acknowledgement

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