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Tinospora crispa accelerated Wound Healing Potential

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Abstract

Introduction: The skin is considered one of the largest organs in the human body. It fulfills several important functions, including thermoregulation and fluid homeostasis, in addition to immunologic, metabolic, and neurosensory functions. Healing a wound is a complex process with numerous overlapping phases. *Tinospora crispa* which is a traditional medicinal plant used for treatment a lot of diseases.

Objectives: This research was conducted to assess the effect of the *Tinospora crispa* extract on closure rate of wound healing potential, histology of wound area and inflammatory mediators; transforming growth factor-βeta (TGFβ1) and tumor necrosis factor α (TNF-α).

Methodology: Twenty adult Sprague-Dawley rats were used in wound healing evaluation experiment and divided into 4 groups. Experimentally, Two-centimeter-diameter full-thickness skin excision wounds were created on the posterior neck area using round seal. The animal groups were topically treated with 0.2 mL gum acacia as vehicle control group, Intrasite gel as reference group, and 100 and 200 mg/mL of *T. crispa* stem extract, respectively as experimental groups. Granulation tissue was excised on the 15th day and processed for histological and biochemical analysis. Wound healing was evaluated by measuring wound contractions and protein contents in the healing wounds. Cellular redistribution and collagen deposition were assessed morphologically. The sera levels of TGF-β1 and TNF-α were evaluated for all the animals.

Results: Rats dressed with *T. crispa* extract showed significantly accelerated wound healing closure, increased TGF-β1 level, decreased TNF-α levels compared with the rats dressed with vehicle or Intrasite gel. Histology of wound healed area confirmed the results showing remarkable increase in collagen fiber, reduced leucocytes infiltration.

In conclusion: the current study suggests that the ethanol extract of *T. crispa* stem has significant excision wound-healing potential. Topical treatment with *T. crispa* extract improved the activity of endogenous antioxidants and prevented free radical-mediated tissue injury. This extract also played an important role in the inflammation process, and the remodeling phase in wound healing.

Key word: *T. crispa*, wound-healing, TGF-β1 and TNF-α

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الملخص

مقدمة: يعتبر الجلد من أكبر أعضاء جسم الإنسان. يقوم بالعديد من الوظائف المهمة ، بما في ذلك التنظيم الحراري واستتباب السوائل ، بالإضافة إلى الوظائف المناعية والتمثيل الغذائي والوظائف الحسية العصبية. يعد التئام الجرح عملية معقدة مع العديد من المراحل المتداخلة Tinospora crispa .هو نبات طبي تقليدي يستخدم لعلاج الكثير من الأمراض.

الأهداف: تم إجراء هذا البحث لتقييم تأثير مستخلص Tinospora crispa على معدل إغلاق إمكانات التئام الجروح ، و أنسجة منطقة الجرح والوسائط الالتهابية. تحويل عامل النمو βeta (TGFβ1) وعامل نخر الورم.(α (TNF-α

المنهجية: تم استخدام عشرين فأرًا بالغًا في تجربة تقييم التئام الجروح وتم تقسيمها إلى 4 مجموعات. تجريبيًا ، تم إنشاء جروح بقطر 2 سم بسمك كامل على منطقة الرقبة الخلفية باستخدام ختم دائري. تمت معالجة المجموعات الحيوانية موضعيًا باستخدام 0.2 مل من أكاسيا الصمغ كمجموعة تحكم في المركبات ، وجل Intrasite كمجموعة مرجعية ، و 100 و 200 مجم / مل من مستخلص جذع .T تم استئصال النسيج الحبيبي في اليوم الخامس عشر ومعالجته للتحليل النسيجي والكيميائي الحيوي. تم تقييم التئام الجروح عن طريق قياس تقلصات الجروح ومحتويات البروتين في الجروح الملتئمة. تم تقييم إعادة التوزيع الخلوي وترسب الكولاجين شكليًا. تم تقييم مستويات المصل من TGF-β1 و TNF-α لجميع الحيوانات.

النتائج: أظهرت الفئران التي تم ارتداؤها بمستخلص T. crispa تسريعًا كبيرًا في التئام الجروح ، وزيادة مستوىTGF-β1 ، وانخفاض مستويات TNF-α مقارنة بالفئران التي تم تزييفها بالسيارات أو هلام .Intrasite أكد علم الأنسجة في منطقة التئام الجروح النتائج التي أظهرت زيادة ملحوظة في ألياف الكولاجين ، و انخفاض تسلل الكريات البيض.

في الختام: تشير الدراسة الحالية إلى أن مستخلص الإيثانول من جذع T. crispa له إمكانات كبيرة في التئام الجروح. أدى العلاج الموضعي بمستخلص T. crispa إلى تحسين نشاط مضادات الأكسدة الذاتية ومنع إصابة الأنسجة بوساطة الجذور الحرة. لعب هذا المستخلص أيضًا دورًا مهمًا في عملية الالتهاب ومرحلة إعادة البناء في التئام الجروح.

الكلمات المفتاحية : T. crispa :، التئام الجروح ، TGF-β1وTNF-α

Introduction:

There are many kinds of acute skin wounds, including incision wounds, incomplete thickness damages and wounds including a special tissue lack. Dissimilar wound kinds include diverse phase's process of healing to varying degrees. The healing of wounds comprises a series of events. These events occur in an accurate to regulate mode. There is overlapping in the wound phases, but they are described in a linear manner to make it clear. The phases of wound healing include hemostasis, inflammation, cellular migration and proliferation, protein synthesis and wound contraction, and finally the remodeling phase. In the healing of the wound, activation of complicated net cells of blood, tissues, growth factors and cytokines cause increased cellular activity that causes rising metabolic requests for nutrients (David &Heather, 2000).

Many nutrition agents which might enhance healing wound time and results are needed to repair the wound. Epithelial and bone formation require vitamin A, important for immune function. Vitamin C is essential for collagen creation, as a tissue antioxidant and for good functioning of the immune system. The main lipid soluble antioxidant in the skin is vitamin E (Mackay & Miller, 2003). Excess ROS result in the killing of fibroblasts, and skin lipids will become less flexible. Because of this, antioxidants seem to be significant in the effective treatment and management of wounds. Antioxidants diminish these adversative effects of wounds through eliminating products of inflammation (Houghton et al., 2005).

The genus of the Tinospora plant has been known as a traditional medicine in Southeast Asian countries. Tinospora has long been used in India as a medication and in the preparation of a starch known as gilae-ka-sat or as palo. It is a tonic, a diuretic and an antiperiodic. Tinospora crispa, which is abundant in the Philippines, is widely used by the population under the name of makabuhany, which means "You may live". It is used as an antidote and is especially valuable in general weakness, malarial fevers and chronic rheumatism. The whole plant contains the bitter principle colombine, alkaloids, flavonoids and glucoside (Dweck & Cavin, 2006).

A previous study was done to evaluate the nutritional components and mineral content of the stem of T. crispa. Results revealed that T. crispa contains a high moisture content of around 77.9%, carbohydrate content 19.4%, while the percentages of fat, protein, fiber and ash are low. The most abundant elements are calcium and potassium and other trace elements such as silicon, magnesium, chlorine and phosphorus are very low. The results also demonstrated that T. crispa extract has a high antioxidant property. This result was confirmed by the existence of phenolic and flavonoids in the extract as catechin, luteolin, morin and rutin, which are responsible for the high antioxidant activity. Outcomes from this study suggested that T. crispa could be an important source of nutrients and natural antioxidant (Amom et al., 2009). Many previous studies were proved that T.crispa has many biological activities (Ruan et al., 2013). In this study was investigated the wound healing ability of T.crispa.

Methods:

Extraction of plant:

Tinospora crispa are dried stems collected from the Bandar Baru Bangi, Selangor housing area. The two plants were authenticated by Mr. Shamsul Khamis, a botanist at the Institute of Bioscience (IBS), University Putra Malaysia (UPM). A voucher specimen (KLU 45568) for Tinospora crispa were preserved in the herbarium of IBS, UPM. The dried plant was crushed and the powder (100 g) was placed in a conical flask and soaked in 900 ml 95% of ethanol for three days at room temperature (30 ± 2 Co). The suspension was shaken from time to time to allow the powder to dissolve completely in the ethanol and the color to change to dark brown. The mix was filtered after three days using a filter paper (Whitman, 185 mm) and the extract was distilled under reduced pressure in a rotary evaporator (Buchi, Switzerland). The extract was maintained at -20ºC prior to usage (Trusheva et al., 2007).

Wound healing ability of plant extract

The animals used were healthy adult Sprague Dawley female rats weighing 250 gram to 300 gram. All rats were housed individually and maintained under standard conditions of humidity (50-60%), temperature (22 ± 3°C) and light (12h light: dark cycle) and fed

purina chow diet and water ad libitum. For the experiment, each rat was caged alone and fasted before treatment (food, but not water, was withdrawn overnight). Throughout the experiments, all criteria for taking care of animals provided by the National Academy of Sciences and defined in the "guide for the care and use of laboratory animals" are applied (Clark et al., 1997). The vehicle used during the study was gum acacia dissolved in normal saline with ethanol extract. Two g of gum acacia was dissolved in (100 ml) of normal saline. Ten ml of solution was used to dissolve 1 g and 2 g of ethanol extract and each ml of solution contained 100 mg or 200 mg of extract (Shetty et al., 2008). The adult SD rats were randomly divided into six groups, with five rats in each group. Each rat was housed separately. The animals were anesthetized, their skins were shaved using an electrical shaver and they were disinfected with 70% alcohol. A uniform wound area 2.00 cm in diameter was excised from the nape of the neck of all the rats using a round seal, as described by Morton et al. (Morton & Malone, 1972) . Incision of the muscle layer was avoided and skin tension was kept constant during the procedure. All of the rats were treated twice a day. The vehicle control group with 0.2 ml gum acacia. The reference drug group with 0.2 ml Intrasite gel. One of the treated groups with 0.2 ml of 100 mg/ml plant extract. The other treated group with 0.2 ml of 200 mg/ml plant extract.

The contraction of the wound area was measured on 0 day, 5th day, 10th day, and 15th day. On the 15th day, all of the rats were administered high doses of anesthesia and the skin from the healed wound area was excised to obtain homogenate tissue for histopathological examination. Blood samples were also obtained to measure other parameters. Measurement of TNF-α and TGF-β1 by ELISA. The blood samples of the rats were collected in tubes. The blood was allowed to clot for 30 min at 25^{o}C, then centrifuged at 2000xg for 15min at 4 ^{o}C using refrigerated centrifuge then the serum was collected and preserved at -80^{o}C until use. The measurement of rat TGF-β1 was by ELISA kit (Abnova, Cat#. KA0279; version: 04) and rat TNF-α by ELISA kit (Thermo Scientific, Cat# .ER3TNFA). The assays were performed as per the detailed instructions of the manufacturer (appendix II). The sensitivity limit of these assays was TGF-β1 is 7.8 pg/ml and <15pg/ml for TNF-α.

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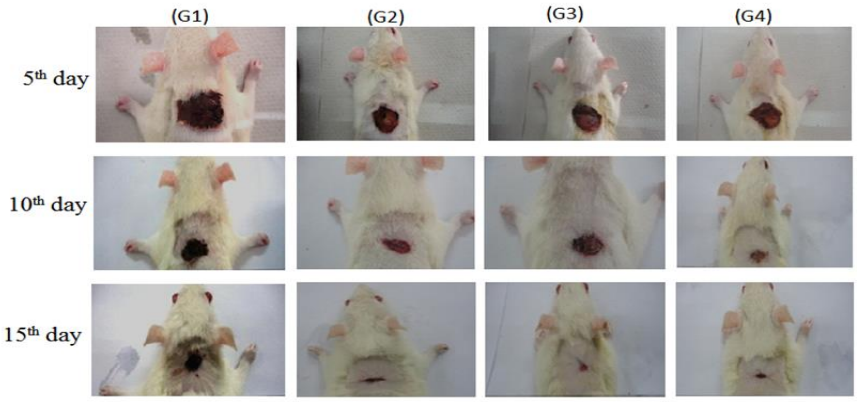

Figure 1: Effect of *T. crispa* extract treatment on macroscopic appearance of excision wound healing area. (G1) 0.2 mL vehicle (gum acacia); (G2) 0.2 mL Intrasite; (G3) 0.2 mL of *T. crispa* 100 mg/mL ;(G4) 0.2 mL of *T. crispa* 200 mg/ml.

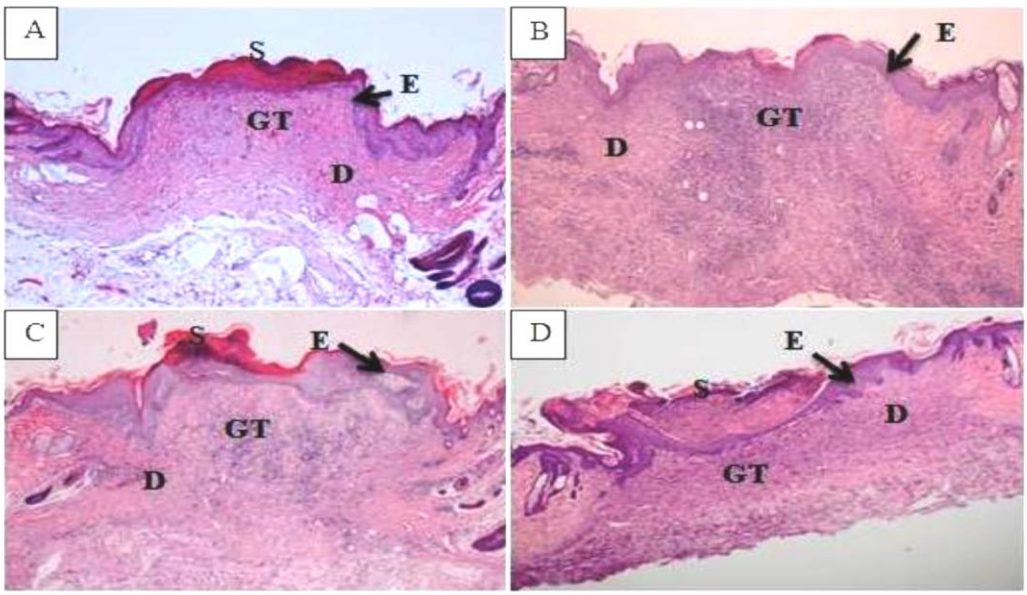

Figure 2: hematoxylin and eosin-stained sections of the wound on the 15th day after wounding in rats. (A) 0.2ml of vehicle, gum acacia in normal saline. (B) 0.2ml of Intrasite gel. (C) 0.2ml of T. crispa (100mg/ml). (D) 0.2ml of T. crispa (200mg/ml). S = Scab, E = Epidermis, GT = granulation tissue. (Magnification 20x)

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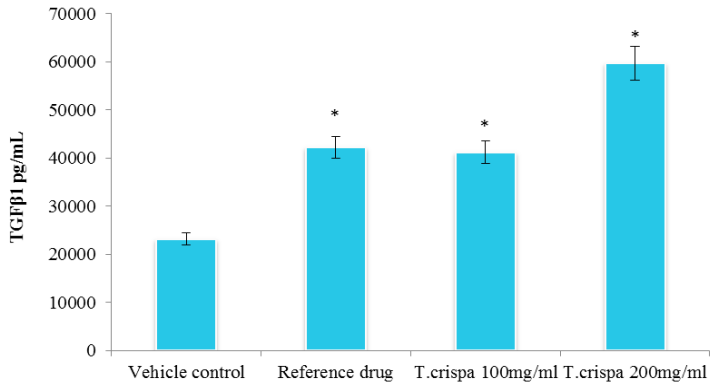

Figure 3: Effects of T.crispa on TGF-β1 serum level in the treated wounded rats. Values are presented as mean ± S.D., (5 rats/group) * Significant ($P \leq 0.05$) compared to the vehicle control.

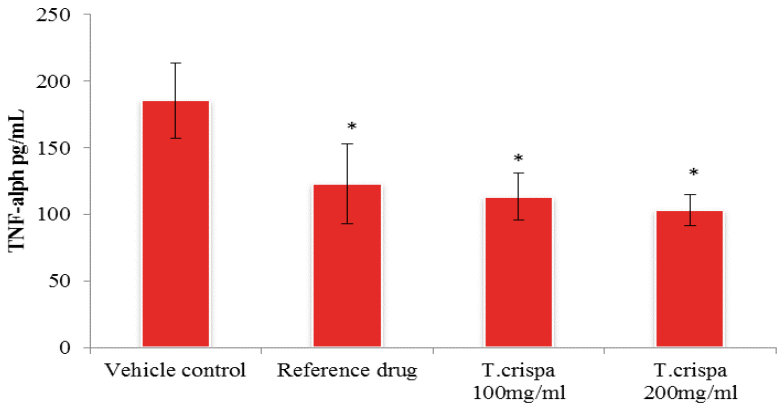

Figure 4: Effects of T.crispa on TNF-α serum level in the treated wounded rats. Values are presented as mean ± S.D., (5 rats/group) * Significant ($P \leq 0.05$) compared to the vehicle control.

Discussion

The main finding of this study is that the topical application of T. crispa extracts to skin excision wounds in rats resulted in an improved wound contraction rate and a serious reduction in healing time compared to the control group. This result may be attributed to the enhanced progression of the wound healing process and the exhibition of noticeable wound margin dehydration as a result of tissue regeneration. The histological evaluation of wound areas in treated groups confirmed the display of increased cellular infiltration, angiogenesis, increased fibroblasts and collagen deposition**.** The underlying mechanisms of topical T. crispa action in the wound area which are caused by the chemotactic influence of the plant extract may attract inflammatory cells. The mitogenic activity of the plant extract may increase cellular proliferation and contribute significantly to the healing process. T. crispa treated groups had significantly smaller wound areas on the 15th day after wounding compared to the vehicle control group. Reactive oxygen species (ROS) are deleterious to the wound healing process because of their harmful effects on cells and tissues. Absorbable synthetic biomaterials are degraded by ROS (Aliyev et al., 2004).

The presence of these antioxidant and anti-inflammatory properties could be one of the factors that contribute to the wound-healing potential of the T. crispa extracts. The topical application of T. crispa restored the activities of these antioxidant enzymes which may help to avoid the deleterious effects of free radicals. Several previous studies have reported the utilization of the antioxidant ability of plant and ability to induce the activity of antioxidant enzymes in the prevention or treatment of a variety of diseases such as cancer, diabetes, atherosclerosis, hyperglycemia, and hypercholesterolemia besides having anti-inflammatory ability (Mohammed et al., 2012).

TGF-ß1 plays an important role as an inflammatory mediator in the initiation of wound healing by activating and stimulating the macrophage to secrete cytokines that act as fibroblast growth factors, platelet derived growth factors, TNF-α and interleukin1. The proliferative phase TGF-β1 level was elevated as secreted by the macrophage, T lymphocytes and platelets. TGF-ß1 is believed to be a major control signal regulating fibroblast functions. TGF-β1 has three effects on extracellular matrix precipitation: it enhances the gene transcription of collagen, fibronectin and proteoglycans, which are

important in the production of matrix proteins, inhibits the production of proteases in charge of matrix breakdown and stimulates the metalloprotease inhibitor (Diegelmann & Evans, 2004). This was also observed in our study with a significant elevation in the level of TGF-ß1 in groups treated with T. crispa extract compared to the vehicle control group and a significant reduction in the level of TNF-α on day 15 after wounding. These results clearly indicate that the effect of both plants in accelerating wound healing may be due to the effect of the rapid movement to the proliferative phase and the shortening of the inflammatory phase to enhance wound contraction. To provide supplementary evidence for this suggestion, previous studies have reported that during the second and third week of healing, fibroblasts start to adopt myofibroblast phenotype properties via large parcels of actin containing microfilaments which are organized along the cytoplasmic cover of the plasma membrane, establishing cell to cell and cell matrix linkages (Hinz et al., 2004; Welch et al., 1990).

In conclusion: the current study suggests that the ethanol extract of *T. crispa* stem has significant excision wound-healing potential. Topical treatment with *T. crispa* extract improved the activity of endogenous antioxidants and prevented free radical-mediated tissue injury. This extract also played an important role in the inflammation process, and the remodeling phase in wound healing.

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