Datura metel Seed Nano Chitosan Extract Gel's Effect On Angiogenesis Wound Healing Of Wistar Rat

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ABSTRACT

Background: *Datura metel possesses anti-inflammatory properties useful for treating oral mucosal injuries common in children, where healing relies on angiogenesis. Nano chitosan technology addresses herbal bioavailability issues. This study investigated the effect of 1% Datura metel seed nano chitosan extract gel on angiogenesis in Wistar rats*

Methods: *Eighteen Wistar rats were divided into three groups: 1% Datura metel seed chitosan extract, 1% nano chitosan extract, and a control group. Gels were applied to mandibular gingival wounds with assessments on days 3 and 5. Histological angiogenesis counts were analyzed using two-way ANOVA ($p<0.05$).*

Results: *Significant differences in angiogenesis counts were found among the chitosan extract, nano chitosan extract, and positive control groups. Furthermore, variations in angiogenesis counts were observed between day 3 and day 5 across all groups.*

Conclusion: *The 1% Datura metel seed chitosan extract gel demonstrated a greater effect in triggering angiogenesis compared to the 1% nano chitosan extract and the positive control.*

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INTRODUCTION

Flora has good potential in treating and managing certain diseases. It has been used by several countries to treat disease conditions. In the last few decades, an increased interest has been seen in the study of natural ingredients that can be a potential source of useful medicinal substances. The medicinal importance of flora can be credited to their bioactive phytochemical components which can lead to certain physiological action in living organisms.[1] Indonesia has natural diversities, and these are widely used in the health sector with the emergence of technological sophistication. *Datura metel* has benefits as herbal medicine as anti-inflammatory, antibacterial, anti-asthmatic, sedatives, analgesic, and antioxidant.[2, 3, 4] *Datura metel* main components are alkaloids (scopolamine, hyoscyamine, atropine[3]), withanolide, flavonoid, sesquiterpene, lignan, dan phenolic acids.[4]

Injuries of mucosal tissue of the child's oral cavity often occur in the children's oral care and treatment. Loss of epithelial integrity in the oral cavity will trigger the process of wound healing, which is characterized by the start of hemostasis, inflammation, proliferation, and remodeling processes. Excessive or prolonged inflammation can have detrimental effects on wound healing physiologically and psychologically.[5] The proliferative phase is marked by angiogenesis, extracellular matrix synthesis and re-epithelialization.[5,6] In addition to contributing to the initiation of the inflammatory response by interacting with leukocytes, microvascular endothelial cells are of utmost importance for the proliferative phase. In order to re-oxygenate the tissue and deliver the necessary nutrients to the newly forming granulation tissue in the wound, angiogenesis, the development of new capillaries from previously existing capillaries, is essential.[7] Three to five days post-injury, new capillaries can be seen in the wound bed in the form of granulation tissue, which serves as a matrix for vascular proliferation and migration of new fibroblasts and collagen.[8]

The wound healing process can stop at any phase, triggering the formation of chronic non-healing wound.[5] The emergence of herbal medicines practice nowadays seems increasing globally, especially in China, India and Indonesia. Consumption of herbal medicines has been a fashion that is on the rise because they have pharmacological activity in almost all ailments and have mild side effects. The most frequent problems of herbal medicines are bioavailability, solubility, absorption of active substances, and low stability.[9] In addition, active components in herbal medicines tend to have difficulties in penetrating the lipid membranes of body cells due to having large molecular sizes and low water solubility, resulting in poor absorption and bioavailability. Problems like these can cause many floras that have potential active substances that cannot be used in vivo even though in vitro shows good results.[10] To overcome these problems, the development of technology used for the formulation of herbal medicines is carried out, called nanoparticle technology.[9]

The use of nanoparticles has become the latest trend in research for drug delivery systems. Nanometer-sized particles or globules have unique physicochemical properties when compared to micrometer or millimeter sized particles. These properties make nanoparticles significantly effective for improving the quality of drug delivery.[11] The nanoparticle size is suitable not only for parenteral application, but also for administration via topical oral, which are non-invasive routes. The development of drug delivery systems by optimizing their pharmaceutical action in vivo is a challenge.[12]

Drug delivery has numerous problems such as bioavailability, solubility, active substances not well absorbed and weak stability of herbal medicine. Datura metel that has a positive angiogenesis phase,

requires the development of nanoparticle technology as a drug delivery. This study was conducted to evaluate the effect of nano chitosan extract gel (1% Datura metel) on wound healing angiogenesis of white Wistar rats.

METHODS

This type of research is laboratory experimental research with Ethical Clearance No.00745/KKEP/FKG-UGM/EC/2021. The ethanol extract of *Datura metel* seeds was processed into 1% *Datura metel* seed nano chitosan extract using ionic gelation method to obtain the nano formulation gel. The research subjects were 18 rats from the Wistar rat strain aged 2.5 – 3 months, male and weighing 200 – 250 g. Rats were injured using 2-mm diameter punch biopsy on the labial gingiva between the mandibular central incisors. Applying gel to the injured area according to each group: 1% *Datura metel* seed chitosan extract gel treatment (group A), 1% *Datura metel* seed nano chitosan extract gel treatment (group B) and using Difflam® gel (group C) in the group positive control. Gel applications were carried out as much as 0.1 g (100 mg) with a frequency of 2 times a day in the morning and evening every day until day 5. Observations were carried out on days 3 and 5. Rats were sacrificed by cervical dislocation, preceded by Ketamine 10% dose of 50 mg/kgBB then Xylazine 2% 5 mg/kgBB. The tissue was fixed with 10% formalin for 24 hours to prevent changes in the tissue. Histological preparations were stained with Hematoxylin-Eosin and angiogenesis was observed and counted using light microscope and *Optilab*® camera with 60x magnification to calculate the amount of angiogenesis in the entire field of view on histological preparations, then summed and averaged. The parameters used in this study were the angiogenesis count formed on day 3 and day 5. The research data were tested using the Kolmogorof-Smirnov normality test ($p>0.05$) and the Levene Test homogeneity test ($p>0.05$). The resulting data were normally distributed and homogeneous, so a two-way ANOVA parametric statistical test was performed with a significance level of 95% ($p <0.05$) followed by the LSD Post Hoc Test to see which treatment groups had significant differences.

RESULT

Angiogenesis in the post-injury wound area showed ongoing wound healing in the proliferative phase. The mean angiogenesis count of 1% *Datura metel* chitosan extract treatment group, 1% *Datura* metel chitosan nano extract, and positive control was presented in Table 1 and illustrated in Figure 3. On day 3, the mean angiogenesis of 1% *Datura* metel chitosan extract group showed the highest result, followed by the 1% *Datura metel* seed nano chitosan extract group and the positive control group. This showed that the angiogenesis activity of the 1% *Datura metel* chitosan seed extract group was faster than the 1% *Datura metel* seed nano chitosan extract group and the positive control group. On day 5, the mean angiogenesis of the 1% Datura chitosan extract group showed the highest results, followed by 1% *Datura metel* nano chitosan extract group and the positive control group.

Table 1. Mean and Standard Deviation Angiogenesis Count Based on Days and Groups Treatment

Treatment Day	N	Treatment Groups	Mean $(x) \pm SD$
Day 3	3	1% *Datura metel* seed chitosan extract	25,33 ± 9,29
	3	1% *Datura metel* seed nano chitosan extract	11,67 ± 5,03
	3	Positive control	10,67 ± 8,50
Day 5	3	1% *Datura metel* seed chitosan extract	44,67 ± 9,50
	3	1% *Datura metel* seed nano chitosan extract	28,33 ± 12,50
	3	Positive control	20,33 ± 11,37

Description:
N : Total subject
SD : Standard Deviation

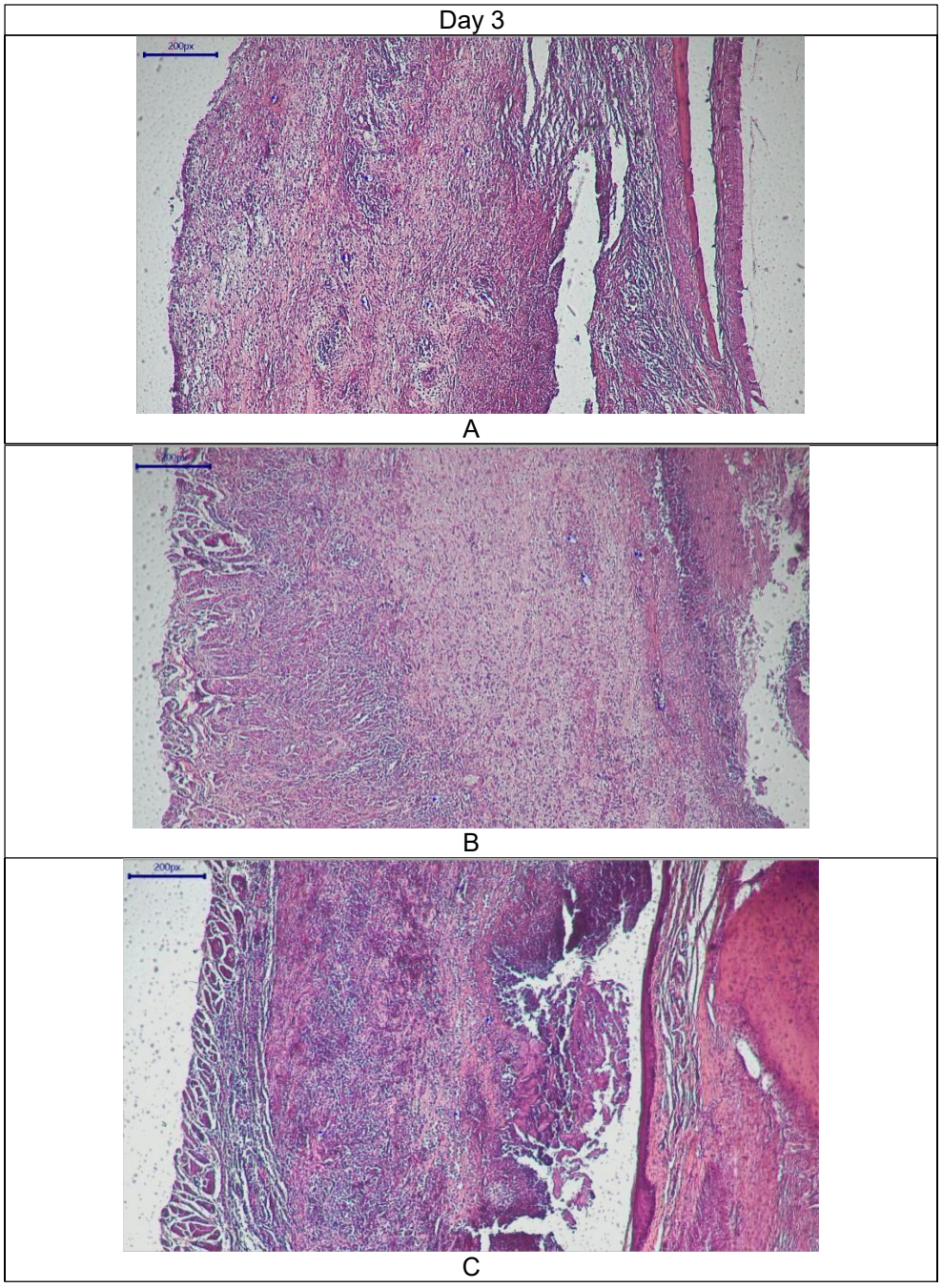
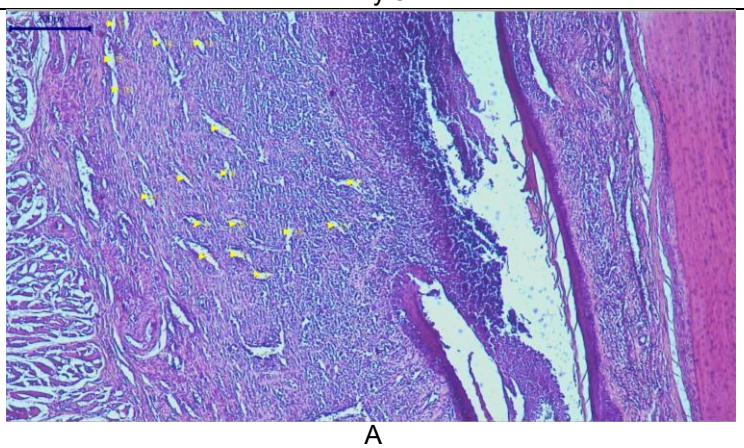
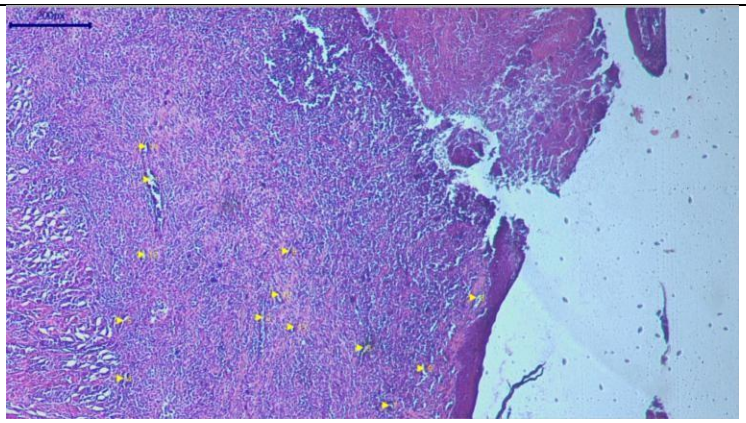


Figure 1. Histological finding of angiogenesis with *Hematoxylin-Eosin* staining with 60x magnification, on Day 3 observation of group A (1% *Datura metel* chitosan extract group), group B (1% *Datura metel* nano chitosan extract group), and positive control group

Day 5



A



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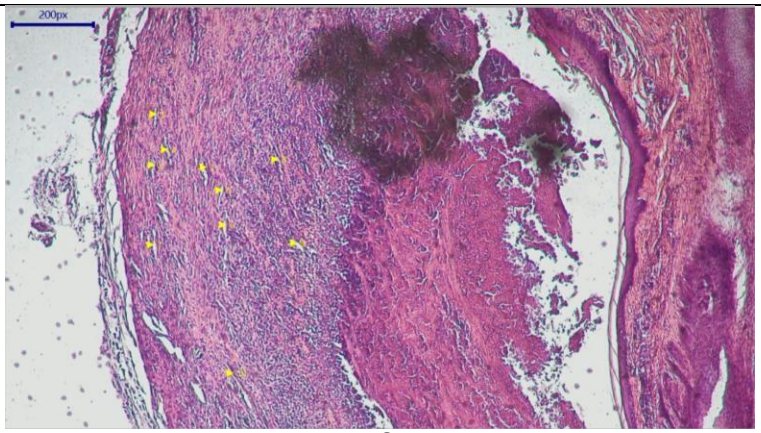

C

Figure 2. Histological finding of angiogenesis with *Hematoxylin-Eosin* staining with 60x magnification, on Day 5 observation of group A (1% *Datura metel* chitosan extract group), group B (1% *Datura metel* nano chitosan extract group), and positive control group

Histological findings of angiogenesis formed on day 3 and 5 are presented in Figure 1 and Figure 2.

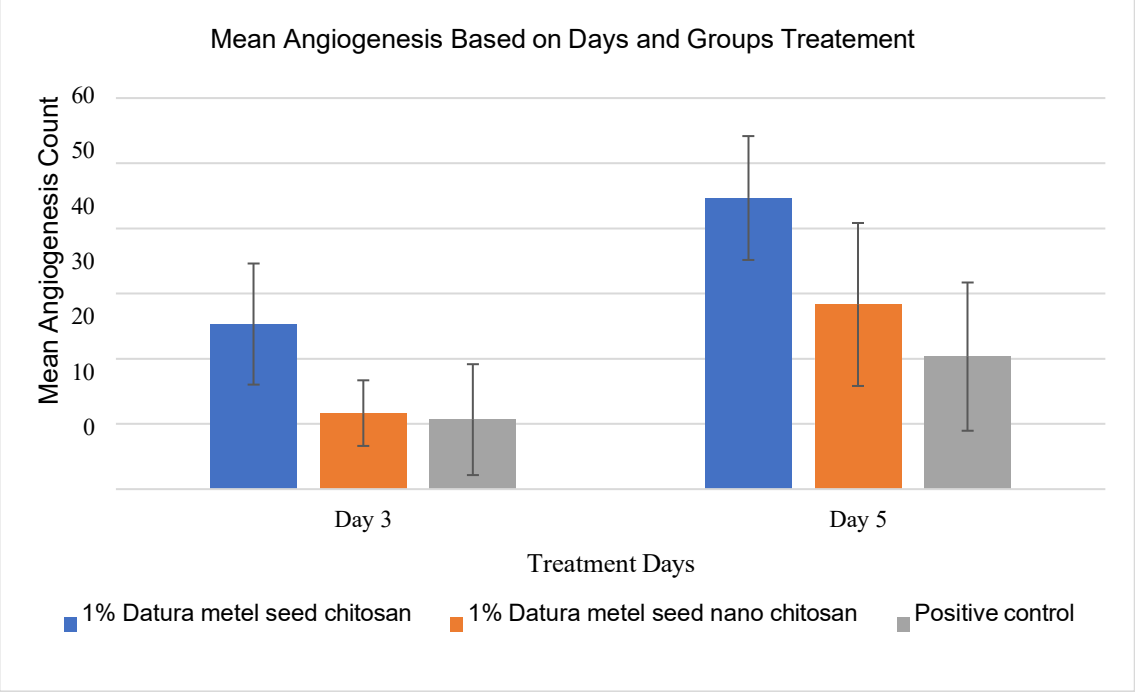

Figure 2. Mean and Standard Deviation Angiogenesis Bar Chart Based on Treatment Days and Treatment Groups

Calculation of the mean significance of the angiogenesis count of the 1% *Datura metel* chitosan seed extract group, 1% *Datura metel* seed nano chitosan extract, and the positive control group on day 3 and day 5 was carried out using the two-way ANOVA (Table 2). Table 2 showed that there was significant difference ($p < 0.05$) in the count of angiogenesis in all treatment groups. This meant that the amount and activity of angiogenesis in 1% *Datura metel* seed chitosan extract group, 1% Datura seed nano chitosan extract group, and positive control group had statistically different results, both on day 3 and day 5.

Table 2. Two-way ANOVA Angiogenesis Count Based on Treatment Days and Treatment Groups

	Type III Sum of Squares	*df*	*Mean Square*	*F*	*Sig.*
Days	1042,722	1	1042,722	11,172	,006
Groups	1251,000	2	625,500	6,702	,011
Days * Groups	74,778	2	37,389	,401	,679

The results of two-way ANOVA in Table 2 also showed significant difference ($p<0.05$) in the count of angiogenesis on day 3 compared to day 5. Comparison of angiogenesis count between day 5 and day 3 showed significant difference in all treatment groups.

Table 3. Post Hoc LSD Angiogenesis Count Based on Treatment Groups

Treatment Group	Treatment Group	*Mean Difference*	*Sig.*
1% *Datura metel* chitosan extract group	1% *Datura metel* nano chitosan extract group	15.00*	.020
	Positive control	19.50*	.004
1% *Datura metel* nano chitosan extract group	1% *Datura metel* chitosan extract group	-15.00*	.020
	Positive control	4.50	.435
Positive control	1% *Datura metel* chitosan extract group	-19.50*	.004
	1% *Datura metel* nano chitosan extract group	-4.50	.435

LSD Post Hoc (Table 3) results showed a significant difference at $p<0.05$ for 1% Datura metel seed chitosan extract group when compared with the 1% Datura metel seed nano chitosan extract (sig. 0.02) and positive control (sig. 0.004), while 1% Datura metel seed nano chitosan extract group was not significant to the positive control group (sig. 0.44).

DISCUSSION

Results showed that the mean angiogenesis for 1% *Datura metel* seed chitosan extract treatment group on day 3 was higher than the 1% *Datura metel* seed nano chitosan extract group and the positive control group. *Datura metel* has role in the process of proliferation of new blood vessels. This is in line with research which stated that the anti- inflammatory activity of ethanol extract showed significant activity at a dose of 200 mg/kg compared to positive control. [13] Significant results were also found for the anti-inflammatory properties of Datura leaves and seeds in the inflammatory phase of the wound healing process compared to the control group. Triterpene and phytol components in this study are thought to have anti-inflammatory properties in the inflammatory phase.[14]

Formation of new vascularization through angiogenesis is considered as important aspect in wound healing. As the wound healed, there is a period of rapid and vigorous growth of capillaries that compose a capillary network many times that of normal tissue. The capillaries that form over time regress causing the final vascular density to be approximately the same as that of normal skin. This new capillary

is needed to carry nutrients, immune cells, and oxygen for wound healing.
(15) This was in accordance with the results of this study which showed that the count and activity of angiogenesis increased in all treatment groups. Angiogenesis on day 3 and day 5 showed increasing in number and activity.

The anti-inflammatory activity of *Datura metel* was suspected from the content of phenolic acids and lignans.[4] The dose- dependent methanol extract of Datura showed significant anti-inflammatory effects in vitro. It was further stated that evaluation of *Datura metel* herbal medicine in vitro and in vivo showed a better initial wound healing process compared to controls.[16] This was in accordance with the results of this study which showed proliferation of angiogenesis on day 3 in the 1% *Datura metel* chitosan extract group and 1% *Datura metel* nano chitosan extract group. Fibroblast cell migration plays an important role in the initial phase of the wound healing process. The ethanol extract of Datura flowers could shrink the incision and excision wound after being induced with *Datura metel* ointment. Wounds treated with *Datura metel* ointment epithelialized more quickly. The granulation tissue of the wound is predominantly made up of fibroblasts, collagen, edema, and new blood vessels. Flavonoids and terpenoids present in the study were presumed to be directly involved in the wound contraction and enhanced re- epithelialization.[17]

Optimal wound healing is vital in terms of survival, as the re-establishment of the integrity of the skin prevents infection and dehydration. 18, 19 Wound healing occurs naturally within days and sometimes even weeks. Topical preparations including ointments, gels, or wound dressings may be applied to wounds to prevent risk of infection and promote wound healing. Gels are often used as they are easy to apply and wipe away, and have increased percutaneous absorption over other types of drug preparations.[19]

Endothelial cells have important role during the wound healing process[18, 20, 21, 22], including the formation of new blood vessels, allowing leukocyte migration, transporting of oxygen and nutrients to the wound area, and secreting biologically active substances.[20] Acute inflammation in response to tissue injury was a series of overlapping, well-orchestrated and strictly sequenced phases such as hemostasis, inflammation, proliferation and remodeling.[18] New capillaries that became visible in the wounded area at day 3-5 were accompanied by granulation tissue which served as a matrix for the proliferation of blood vessels and migration of fibroblasts and new collagen.[8] The findings revealed that the proliferative stage of neovascularization began from day 3 and proliferated on day 5.

Based on statistical tests, there was significant difference in the count of angiogenesis in all treatment groups on day 3 and day 5. This was caused by the process of endothelial proliferation appeared to increase rapidly to achieve a density of new blood vessels that far exceed the normal intact tissue. The regression phase of wound healing is when most of the new blood vessels undergo apoptosis, and the capillary bed is pruned back to a normal vascular density. The process of regression is strictly regulated and involves the selective apoptosis of the newly formed capillaries. The initiation of angiogenesis was regulated by soluble factors, particularly vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, platelet derived growth factor (PDGF), and members of the Transforming Growth Factor Beta (TGF-β) family.[18]

Microvascular endothelial cells were major participants in the proliferative phase, aside from the inflammatory response through their interactions with leukocytes. Angiogenesis was needed to restore oxygenation and to nourish the newly forming granulation tissue in the wound.[7] Adequate vascularization

of almost all multicellular tissues is required to ensure survival. Functional restoration of capillary nets is particularly crucial for the survival of tissues following injury. Angiogenesis is absolutely required for wound healing, and normal wound healing demonstrates robust capillary growth, which is followed by programmed capillary regression.[18] Since angiogenesis is needed in wound healing, the required nature of this process is advantageous for various clinical situations to attain wound closure.[22]

The wound healing potential of alcohol *Datura metel* extract in vivo on rat burns wound was carried out using topically applied D*atura metel* ointment 10% w/w. The chemotactic effect was further enhanced with Datura metel extract resulting in the recruitment of inflammatory cells to the wound site and observations were made by using hematoxylin- eosin staining. The increase in cellular proliferation could probably be due to mitogenic effect of the Datura extract. The matrix metalloprotease (MMP), such as MMP 9, were also expressed on early days of wound healing for fibrin lysis resulting in the formation of peptides that had angiogenic and chemotactic properties.[23] This study was conducted until day 5, so further research is needed for with a longer duration to determine the proliferative phase which will continue into the wound healing maturation phase.

Some reasons that might cause 1% *Datura metel* seed chitosan extract gel to trigger more angiogenesis compared to the 1% *Datura metel* seed nano chitosan extract gel (Figure 3) were the total flavonoid content and post-manufacturing stability of the 1% *Datura metel* seed nano chitosan extract. Phytochemical tests have been conducted to determine the content of secondary metabolites using the UV-vis spectrophotometry method. The results showed that the total flavonoid content of the 1% *Datura metel* chitosan seed extract gel was at 748.33 mg/kg, while the total flavonoid content of the 1% *Datura metel* seed nano chitosan extract was at 445.43 mg/kg. The content of flavonoids in the 1% *Datura metel* chitosan seed extract was higher than the 1% *Datura metel* seed nano chitosan extract, this was probably due to the encapsulation effect in the form of nanoparticles which had cross-links between chitosan and TPP (tripolyphosphate).[24] Nanoparticles were also unstable and had tendency of aggregation. The aggregation of nanoparticles with each other would cause larger size of the nanoparticles.[(25)] The stability of the 1% *Datura metel* seed nano chitosan extract in this study could not be determined because zeta potential was not measured.[26] Storage conditions could affect storage stability such as temperature solution, pH and ionic strength of the nanoparticles.[(27)] Researchers paid little attention to the temperature and pH of the nano-solution in this study so that there was a possibility of changing the storage stability which then led to a decrease in the content of flavonoids which ultimately led to less angiogenesis in the 1% *Datura* metel seed nano chitosan extract group compared to the 1% *Datura metel* seed chitosan extract group. This did not diminish the benefits of chitosan nanoparticles as a solution for herbal drug delivery systems.

Particle size analyzer (PSA) test showed that the particle size of the *Datura metel* seed nano chitosan extract was at 142 nm. This shows that the material used in this study was truly nano sized. The size of the particles formed met the requirements because the size of nanoparticles that was good drug delivery systems was <300 nm.[28] These nanoparticle preparations after one year's storage can still be diluted back into a good colloidal solution and still have in vitro and in vitro properties.[29]

Nanotechnology is therefore suggested to be used for herbal medicines due to various reasons. For instance, side effects with the presently available formulations, non- compliance to the edications by the patients due to the available formulations employing large doses and being ineffective, lack of target specificity of the existing formulations for multiple chronic diseases.[30] With the presence of

nanotechnology, the bioavailability and absorption of active ingredients can be increased due to an increase in the surface area of the particles and solubility.[31]

CONCLUSION

The results showed that the angiogenesis counts of the positive control group, 1% Datura metel seed chitosan extract, and 1% Datura metel seed nano chitosan extract differed considerably ($p<0.05$). The outcomes of the positive control group and the 1% Datura metel seed nano chitosan extract were comparable. Additionally, there were variations in the angiogenesis count across all treatment groups between day 3 and day 5.

ACKNOWLEDGEMENT

Nothing to declare.

CONFLICT OF INTEREST

Nothing to declare.

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