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Powerful benefits of metel (*Datura metel* L.) amethyst cultivar seed extract nano-chitosan gel for gingival wound healing

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Abstract

Wound healing is a complex process in which the body naturally repairs damaged tissues, and restores their structure and function. Amethyst (purple) cultivar of metel (*Datura metel* L.) seed contains an alkaloid that accelerates wound healing by stimulating fibroblast proliferation and migration. Furthermore, nanotechnology can be utilised with mucoadhesive chitosan polymers and gel preparations to enhance the bioavailability of drugs. The present work aimed to investigate the impact of a nano-chitosan gel containing amethyst seed extract (ASE) on the fibroblast count during gingival wound healing. The present work involved 36 male Wistar rats (*Rattus norvegicus*) aged between three and four months, and divided into four groups: base gel (negative control), Aloclair® gel (positive control), ASE chitosan gel 10%, and ASE nano-chitosan gel 10%. All subjects underwent a 2 mm punch biopsy to their mandibular labial gingiva, and the gels were applied twice a day (morning and evening). Three rats from each group were sacrificed on days 3, 5, and 7, and stained with Haematoxylin Eosin. The fibroblasts were counted using a 40× objective lens magnification microscope. The data were analysed using the Two-way ANOVA test with a confidence level of 95%. The results indicated a significant difference ($p < 0.05$) between the formulations and the base gel. In conclusion, the ASE nano-chitosan gel increased the fibroblast counts during gingival wound healing.

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Introduction

Wound healing is a natural process by which the human body repairs damaged tissues, and restores their structure and function (Nofikasari *et al*., 2016; Rachmanita *et al*., 2019). It consists of four important phases, namely haemostasis, inflammation, proliferation, and remodelling, which overlap with each other. The proliferation phase begins about three to ten days after the wound occurs. This phase is further divided into three stages: re-epithelisation, angiogenesis, and the formation of granulation tissue (Theoret and Schumacher, 2017).

The proliferation phase is dominated by fibroblasts, and their products are collagen and extracellular matrix. Fibroblasts migrate and proliferate in response to growth factors such as Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor (TGF), and fibronectin (Masir *et al*., 2012). Fibroblast Growth Factor (FGF-2) or basic FGF (bFGF) can increase fibroblast migration, and stimulate the proliferation of fibroblasts (Braund *et al*., 2009). The increased number of fibroblasts will increase the amount of collagen, and speed up the wound healing process. Fibroblasts will also differentiate into myofibroblasts that will contract wounds to reduce the size of the wound (Reinke and Sorg, 2012).

Metel (*Datura metel* L.) is a common perennial shrub in Indonesia with white, yellow, red, and purple (amethyst) cultivars. Amethyst offers multiple medicinal benefits, including wound healing, asthma relief, and can act as an analgesic, anaesthetic, sedative, hallucinogen, and anti-spasmodic agent

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(Alabri *et al*., 2014). Similar to other natural resources that have pharmacological effects related to their chemical components (Fawwaz *et al*., 2021; 2024; Azizah *et al*., 2022), amethyst also possesses dominant chemical components that underlie its pharmacological activity. Amethyst contains alkaloids, flavonoids, saponins, and tannins, which function as anti-inflammatory, antimicrobial, antioxidant, and antifungal agents (Alabri *et al*., 2014; Shekhar *et al*., 2017). Alkaloids present in plants can accelerate wound healing by acting as bactericidal agents, promoting angiogenesis, and increasing elasticity (Priya *et al*., 2002; Lai *et al*., 2011; Shekhar *et al*., 2017). Furthermore, alkaloids can enhance the migration and proliferation of fibroblasts through a synergistic mechanism with bFGF (Tsala *et al*., 2013).

Medical research in the field of nanotechnology is expanding rapidly. Nano-formulations have been found to accelerate drug delivery systems due to their small size, which increases surface area, enhances biodegradability, and improves bioavailability, allowing for controlled and sustained drug release (Jong and Borm, 2008; Pal *et al*., 2011). Nanoparticle manufacturing can be achieved by adding polymers as coating materials, with chitosan being a popular choice due to its non-toxic, biocompatible, and biodegradable properties (Debnath *et al*., 2011). Chitosan, derived from shrimp or crab shells, also exhibits mucoadhesive properties that can enhance topical drug delivery, thereby optimising drug release (Adiana and Syafiar, 2014; Fawwaz *et al*., 2018; 2019). Drugs with gel form that also possess mucoadhesive properties are advantageous as they have a semisolid form with a net-like matrix structure, which makes them more stable, less sticky, and easier to clean (Verma *et al*., 2013; Patil *et al*., 2019).

Nanotechnology offers several unique advantages in enhancing the wound healing process. Some nanomaterials can stimulate the proliferation and migration of skin cells, including keratinocytes and fibroblasts, which are crucial for wound closure and tissue regeneration. Nanotechnology can aid in the regeneration of skin and other tissues by delivering growth factors, such as vascular endothelial growth factor (VEGF) and FGF, in a controlled and localised manner (Fathi-Achachelouei *et al*., 2019).

Nanotechnology products currently used for wound healing include nanofiber scaffolds and liposomes. The nanofiber scaffolds provide a scaffold for cell growth and facilitate tissue regeneration in wounds. Additionally, liposome-based drug delivery systems can encapsulate anti-inflammatory agents or growth factors, ensuring their controlled release at the wound site, thus enhancing tissue regeneration (Ovinuchi *et al*., 2024).

Therefore, the present work aimed to investigate the effect of amethyst seed extract (ASE) nano-chitosan gel on the number of fibroblasts during gingival wound healing. The present work would provide information on the application of ASE nano-chitosan gel during gingival wound healing, and produce useful innovations in the field of health sciences, by offering a nanoparticle model based on chemical components from natural materials as active substances.

Materials and methods

Preparation stage

The present work was conducted with ethical clearance from the Ethics and Advocacy Unit of the Medical and Health Research Commission at the Faculty of Dentistry, Universitas Gadjah Mada, Indonesia. The plants used in the present work were identified by expert from the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada.

Amethyst seed extract

The extract was prepared at the Laboratory Unit II, Faculty of Pharmacy, Universitas Gadjah Mada using the maceration method. The amethyst seeds were dried in the oven for 2 h, then mashed and macerated with 70% ethanol. Stirring was conducted for the first 3 h, followed by a waiting period of 24 h. Ethanol was removed using a rotary evaporator, and concentrated in a water bath to obtain a viscous extract of amethyst seeds.

Qualitative and quantitative assays

A qualitative test was conducted at the Laboratory Unit II, Faculty of Pharmacy, Universitas Gadjah Mada using the Thin Layer Chromatography (TLC) method to detect the presence of alkaloid compounds in ASE. The Retardation Factor (Rf) was calculated to determine the presence of alkaloids. The TLC method used a mobile phase with a composition of toluene, ethyl acetate, formic acid (70:20:10). The extract was dipped onto a thin plate of silica gel (F_{254}),

and the mobile phase was used to move the alkaloids present in the extract up to the higher plate. Rf calculation was performed on a thin plate of silica gel (F_{254}). Additionally, a quantitative test was carried out by inserting samples of ASE in the Integrated Research and Testing Laboratory I, Universitas Gadjah Mada to obtain the levels of alkaloids in the extract.

Nano-chitosan formulation

The formulation of nano-chitosan using the ionic gelation method was conducted on the obtained extract. A solution of chitosan was prepared by dissolving 0.2 g of chitosan in 100 mL of 1% glacial acetic acid, which was then mixed with the 10% extract. The extract mixture was added dropwise to a sodium tripolyphosphate solution (0.2 g of sodium tripolyphosphate in 100 mL of aqueous solution) while stirred using a homogeniser at 15,000 rpm for 30 min. The nano-chitosan formulations created were then tested using a Particle Size Analyser (PSA) at the Faculty of Dentistry, Universitas Gadjah Mada. The particle sizes were within the range of 1 – 1,000 nm.

Gel preparations

Preparations of ASE nano-chitosan gel were made in the Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Gadjah Mada. The gel base was made by developing Sodium Carboxymethyl Cellulose (CMC) in 10 mL of water at 80°C. ASE nano-chitosan solution was mixed on the gel base, and glycerine, propylene glycol, and methyl parabens were added. The gel was stirred until homogeneous.

In vivo study

The *in vivo* study was conducted at the Integrated Research and Testing Laboratory IV, Universitas Gadjah Mada. Animal treatment was given to 36 Wistar male rats (*Rattus norvegicus*) aged three to four months. The subjects were divided into four groups: base gel (negative control), Aloclair® gel (positive control), ASE chitosan gel, and ASE nano-chitosan gel. The subjects were first anesthetised with ketamine (100 mg/kg) and xylazine (4 mg/kg), then a 2 mm diameter biopsy punch was performed on the gingiva of the central incisors of the mandible. Each gel was applied twice a day (morning and evening). Three rats from each group were sacrificed on days 3, 5, and 7 by cervical dislocation, preceded by anaesthesia with a ketamine dose of 100 mg/kg and then xylazine at 4 mg/kg. The sacrificed rats had cuts made on the treated jaws. The samples were fixed in 10% formaldehyde for 24 h to prevent tissue changes. The samples were stained with Haematoxylin-Eosin to create a histological slide. The histological slides were observed with a 40× objective lens magnifying microscope and an OPTILAB® camera, assisted by the Image Raster software®. The observation revealed fibroblasts shaped like stellate cells with a purple nucleus and pink cytoplasm.

Statistical analysis

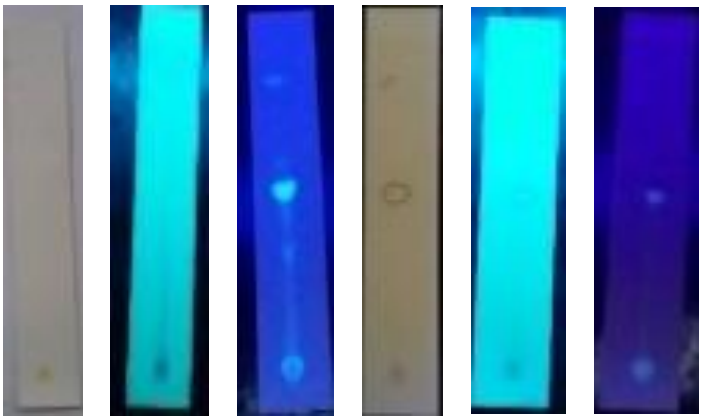
The data obtained were quantitative data measured on a ratio scale, allowing for the use of parametric statistical tests. First, the data were analysed for normality using the Shapiro-Wilk test, and for homogeneity using the Levene's test. Following this, a two-way ANOVA was conducted with a significance level of 95%. Significant findings led to a post hoc test using the Least Significant Difference (LSD) to determine the average differences in fibroblast cell count between groups and observation times on days 3, 5, and 7.

Results

TLC test results showed an Rf value of 0.56 with a mobile phase consisting of toluene, ethyl acetate, and formic acid (70:20:10). Rf alkaloids under UV_{254} appeared as dark spots, while they exhibited strong fluorescence under UV_{356}. Rf alkaloids with Dragendorff's reagent under light appeared as orange spots, indicating the presence of alkaloids in ASE (Figure 1).

After the qualitative test, a quantitative phytochemical test was conducted to determine the percentage of total flavonoids, phenolics, saponins, and alkaloids in ASE, ASE chitosan gel, and ASE nano-chitosan gel. The ASE nano-chitosan gels contained a higher total alkaloid content than ASE chitosan gels (Table 1). PSA test results indicated that 99.8% of particles from ASE nano-chitosan formulations had a size of 487.8 nm, while 0.2% measured 493.9 nm. This verified that the size of ASE nano-chitosan gels fell within the nano-size range

The histological image results of fibroblasts (Figure 2) were taken from binocular microscopes connected with Optilab®. The average and standard deviation of the number of fibroblasts in the base gel group, Aloclair® gel group, ASE chitosan gel 10%, and ASE nano-chitosan gel 10% showed an increase



(A) **(B)** **(C)** **(D)** **(E)** **(F)**

Figure 1. TLC plate (silica gel 60 F254-Merck) before being sprayed with Dragendorff's reagent: **(A)** visible light, **(B)** UV254, and **(C)** UV356; TLC plate after being sprayed with Dragendorff's reagent: **(D)** Visible light, **(E)** UV254, and **(F)** UV356.

Table 1. Phytochemical components of extract and formulations.

Test material	Total flavonoid (%w/w)	Total phenolic (%w/w)	Total saponin (mg/kg)	Total alkaloid (mg/kg)
ASE	4.01	14.10	4,876.65	3,883.86
ASE chitosan gel	0.32	3.56	1,604.37	305.20
ASE nano-chitosan gel	0.15	1.72	1,318.25	320.15

ASE: amethyst seed extract.

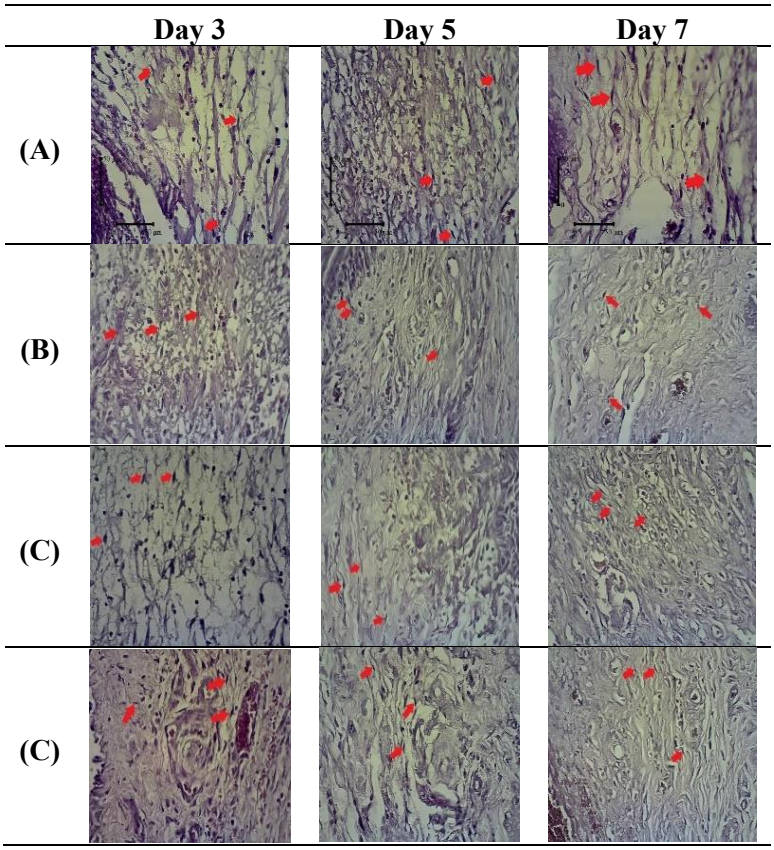


Figure 2. Histological images of fibroblast cells (red arrows) on observations of days 3, 5, and 7 in **(A)** gel base, **(B)** gel Aloclair®, **(C)** ASE chitosan gel 10%, and **(D)** ASE nano-chitosan gel 10%. Scale bar: 50 µm. ASE: amethyst seed extract.

in fibroblast cells from day 3 to day 7 of observation (Table 2).

A parametric test was performed to analyse the data. The analysis of fibroblast data was conducted using the Shapiro-Wilk normality test ($p > 0.05$) and Levene's homogeneity test ($p > 0.05$), which indicated homogeneous data. Further analysis of the fibroblast count data was carried out. The results of the two-way ANOVA test showed that the observation time and the type of gel applied affected the number of fibroblasts, while the interaction between observation time and the type of gel applied did not show significant results regarding the number of fibroblasts. Post hoc LSD test results indicated that the average number of fibroblasts on day 3 was lower than on days 5 and 7. Day 5 showed an increase in the average number of fibroblasts until day 7, which recorded the highest average number of fibroblasts. On day 3, ASE chitosan gel significantly influenced the number of fibroblasts compared to the base gel. On day 5, there was an increase in the number of fibroblasts in all groups, but no statistical differences were found among the groups. On day 7, ASE nano-chitosan gel had a significant influence on the proliferation of fibroblasts compared to the base gel and ASE chitosan gel, but it was similar to Aloclair® gel.

Based on the overall pattern of increase in fibroblasts from day 3 to day 7, the ASE chitosan gel showed that fibroblasts peaked on day 5, then decreased by day 7. In contrast, ASE nano-chitosan gels continued to increase on days 3 and 5, peaking on day 7.

Table 2. Average and standard deviations of fibroblast cell count base gel, Aloclair® gel, ASE chitosan gel 10%, and ASE nano-chitosan gel on days 3, 5, and 7.

Day	N	Group ($\bar{x}$ ± SD)			
		Base gel	Aloclair® gel	ASE chitosan gel	ASE nano-chitosan gel
3	12	34.3 ± 11	48.7 ± 16.3	57.3 ± 8.3	50.7 ± 12.1
5	12	61 ± 11	68.3 ± 8.1	72.3 ± 5.9	62.3 ± 13.1
7	12	66 ± 5.6	74 ± 9.2	69.7 ± 5.1	88.7 ± 7.6

N: research sample; $\bar{x}$: mean; SD ± standard deviation; ASE: amethyst seed extract.

Discussion

Fibroblasts could be observed starting to proliferate from day 3. Fibroblasts in the surrounding area were also stimulated to migrate to the wound site. The migration of fibroblasts to the wound area is influenced by growth factors such as PDGF and TGF-β from platelets and inflammatory cells (Velnar *et al*., 2009). Migratory fibroblasts begin to proliferate and synthesise collagen, while pre-existing fibroblasts in the wound area transform into myofibroblasts (Prasetyono, 2009). The migration and proliferation of fibroblasts continue to increase, peaking on day 7 (Ismardianita *et al*., 2019). The proliferation of fibroblasts produces collagen, proteoglycans, glycoproteins, glycosaminoglycans, laminins, and hyaluronic acid, forming a new extracellular matrix that fills the wound area. The new extracellular matrix aids in cell adhesion and further fibroblast migration to the wound area (Bainbridge, 2013). It also improves the integrity and strength of the wound area, expediting the wound healing process (Siswanto *et al*., 2016). The strength of the extracellular matrix matches that of the surrounding healthy tissue, leading to the onset of cell apoptosis, which normalises the number of cells (Bainbridge, 2013).

A significant difference between the ASE chitosan gel and the base gel group indicated that the ASE chitosan gel influenced the number of fibroblasts. In contrast, the ASE chitosan gel had similar influence to that of Aloclair® gel or ASE nano-chitosan gel. This influence was likely due to the alkaloid content in ASE. *In vivo* research conducted by Mahibalan *et al*. (2016) showed that the application of alkaloid ointment could accelerate wound healing by increasing cell proliferation, epithelialisation, and contraction of wounds. Alkaloids can enhance fibroblast migration through synergy with bFGF (Yalin *et al*., 2005).

bFGF or FGF-2 play crucial role in accelerating wound healing by stimulating fibroblast proliferation, migration, and collagen production (Braund *et al*., 2009). FGF-2 enhances fibroblast migration through the activation of Rat Sarcoma

(Ras)/Mitogen-Activated Protein (MAP) signalling pathways (Song *et al*., 2016). FGF-2 also exhibits a high binding affinity for extracellular matrix fibrin, regulating the wound healing process by stimulating the production of collagen types I and III at lower concentrations; conversely, at higher concentrations, it reduces collagen production (Cornwell and Pins, 2010). Proper regulation of FGF-2 is essential to prevent hypertrophic scars (Song *et al*., 2011). In addition to acting through bFGF, alkaloid compounds such as hyoscyamine, scopolamine, and atropine possess anticholinergic properties by binding to muscarinic receptors. This ability to modulate muscarinic receptors can either stimulate or inhibit rat fibroblast proliferation and wound re-epithelialisation (Gal *et al*., 2009).

The influence of ASE nano-chitosan gel, which was similar to ASE chitosan gel on day 3, might have been due to the healing of new wounds entering the beginning of the proliferation phase, resulting in a non-significant difference in the number of fibroblasts between groups. The proliferation phase occurs between day 3 and day 10, during which the migration and proliferation of fibroblasts are initiated (Theoret and Schumacher, 2017).

On day 5, there was no meaningful difference between all groups. Although there were differences in the number of fibroblasts across the groups, they were insignificant. Wound healing is a biological process that occurs in stages, including inflammatory, proliferative, and remodelling phases. In many instances, significant changes in wound healing may only be observed after a longer period (*e.g*., more than five days). Therefore, the difference between the 3- and 5-day treatments might not have been substantial enough to be clearly noticed, especially if the wound has shown signs of relatively stable healing by the third day. This supported the data on the number of fibroblasts on day 7, which exhibited a significantly different value from that of day 3.

On day 7, there was a significant difference between the ASE nano-chitosan gel and the base gel group. ASE nano-chitosan gels also showed substantial differences compared to ASE chitosan gels, even though both two gels originated from ASE. This was likely due to the extract of amethyst seeds being formulated as nano-sized chitosan gel, which facilitated the absorption process, thereby increasing its bioavailability and resulting in faster, more optimal action. The nano-size also expands the surface area, thus accelerating dissolution and absorption, requiring a lower dose and reducing toxicity levels (Pal *et al*., 2011). The nano-size enhances penetration into cell membranes, and allows for intracellular entry *via* endocytosis by lysosomes and endosomes, followed by degradation along with the release of active substances (Jong and Borm, 2008).

Nanoparticle gel preparations incorporate additional polymers, such as chitosan, which serves as the coating for the active substance. Chitosan is degraded into amino acids, and absorbed by the body, rendering it non-toxic (Debnath *et al*., 2011). Chitosan exhibits good biocompatibility, is biodegradable, and has antimicrobial and mucoadhesive properties (Adiana and Syafiar, 2014). This mucoadhesive property is further enhanced by the gel dosage form. Gel formulations outperform other semisolid preparations and creams due to their non-sticky nature, greater stability, ease of cleaning, and improved drug retention at the application site (Verma *et al*., 2013).

Based on the overall pattern of increase in fibroblasts from day 3 to day 7, the gel ASE chitosan showed that fibroblasts reached a peak on day 5 but then decreased by day 7. In contrast, ASE nano-chitosan gels continued to increase until day 7, and decreased thereafter (Ismardianita *et al*., 2019). The decrease in the number of fibroblast cells indicates the start of the remodelling phase. The remodelling phase can begin on day 8, overlapping with the proliferative phase, and can last until day 21 or even up to one year. During this phase, type 3 collagen is degraded and replaced by stronger type 1 collagen (Prasetyono, 2009).

Fibroblasts will begin to decrease once the extracellular matrix strength has approached or matched the surrounding healthy tissue. At that point, the body initiates apoptosis of cells to reduce the number from the proliferation phase, and return to normal (Bainbridge, 2013). Optimal wound healing features the least fibroblasts at the conclusion of the healing phase, as it re-adjusts the number of cells to resemble those in normal tissues; an excessive number of fibroblasts indicates an ongoing wound healing process (Debnath *et al*., 2011). Overly aggressive healing processes, such as excessive collagen synthesis or unbalanced collagen degradation alongside fibroblasts, can lead to hypertrophic scars or keloids (Prasetyono, 2009). Observations at least until day 14 will reveal a wound healing pattern, as on day 7 the fibroblasts are still at

the peak of the proliferation phase, and begins remodelling on day 8. Based on this, it cannot be concluded that there was better healing between ASE chitosan gel and ASE nano-chitosan, as the decreased fibroblasts that indicated the healing patterns were not yet fully observable.

Conclusion

The usage of ASE nano-chitosan gel has been shown to boost the number of fibroblasts during gingival wound healing. However, additional research with a longer observation period is required. Furthermore, collagen density metrics would supplement the existing data on fibroblasts, whereas more sensitive parameters like Matrix Metalloproteinases (MMP) biomarkers would be advantageous.

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