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Self Nano Emulsifying Drug Delivery System (SNEDDS) Activity of Pegagan (*Centella asiatica* L) Extraction on Zebrafish Caudal Fins Regeneration

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ABSTRACT: *Centella asiatica* L. is a herbal medicine with antioxidant, antihyperglycemic, and wound-healing effects. It contains asiatic acid, madecassic acid, asiaticoside, and madecassoside. Asiatic acid in *C. asiatica* is a non-polar compound, thus it is necessary to overcome the problem of solubility prepared in the SNEDDS formula. This study was conducted to determine the effect of SNEDDS preparations on *C. asiatica* extract on the regeneration of zebrafish caudal fins. The test was conducted using zebra fish divided into 6 groups (n = 8): negative control (amputated without treatment), solvent control (amputated and given SNEDDS without *C. asiatica* extract), treatment group (amputated and given SNEDDS *C. asiatica* extract doses 5, 10, and 20 ppm), and normal group (not amputated and not treated). The regeneration of caudal fins was observed using a stereo microscope for 28 days. The results of the calculation of regeneration areas were analyzed using one-way ANOVA ($p<0.05$). The result showed that the percentage calculation of regeneration areas are 56.19% (negative control); 53.74% (solvent control); 81.09% (5 ppm SNEDDS); 54.04% (10 ppm SNEDDS); and 56.70% (20 ppm SNEDDS). SNEDDS *C. asiatica* extracts 5 ppm have a better regeneration area than others ($p<0.05$). SNEDDS *C. asiatica* extract dose of 5 ppm is proven to have the best wound healing activity in the process of regeneration of zebrafish caudal fins.

KEYWORDS: caudal fin; *Centella asiatica*; wound healing; SNEDDS; regeneration.

1. INTRODUCTION

Centella asiatica L. is a common medicinal plant in India, China, Indonesia, Australia, South Pacific, Madagascar, southern, and central Africa [1]. *C. asiatica* contains components of triterpenoid compounds, alkaloids, saponins, flavonoids, phenolics, tannins, steroids, and glycosides. Components of triterpenoid compounds such as *asiaticoside*, *madecassoside*, and *asiatic acid* are reported to have a wide range of activities such as wound-healing, scar reduction, antioxidant, anti-inflammatory, and antidiabetic [2, 3]. *Centella asiatica* L plant has been used as a traditional herbal remedy in many Asian countries for hundreds of years. One of the substances of *C. asiatica* is the asiatic acid of the triterpenoid group which is believed to have acted as a wound healer [2, 4, 5, 6, 7]. Asiatic acid contained in C. asiatica is difficult to dissolve in water which can cause bioavailability in the body to be low [8]. Thus, it is necessary to develop preparations using nanoparticle technology, namely the *Self-Nanoemulisfying Drug Delivery System* (SNEDDS). The droplet size of SNEDDS usually ranges from 20-200 nm so that the drug can be widely distributed in the gastrointestinal tract which can increase the solubility and absorption of the drug [9]. In addition, SNEDDS has been shown to increase the bioavailability of drugs in the body thereby increasing the effectiveness of treatment [10, 11].

The potential of SNEDDS preparations of *C. asiatica* extract as a wound healer can be known by testing it on zebrafish (*D. rerio*) because zebrafish have more than 70% orthologs with human genes so in the process of wound healing it has almost similarities with mammals [12, 13]. Zebrafish are known to regenerate various

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tissues and organs well in a short time which has a large role in wound healing activities, one of which is on the fins [14, 15]. In addition, zebrafish have a high reproductive ability that makes zebrafish easy to breed [16]. Therefore, this study aims to find out the effect of SNEDDS preparations of *Centella asiatica* leaf ethanol extract on the regeneration of the zebrafish caudal fins.

2. RESULT AND DISCUSSIONS

2.1 *Centella asiatica L.* leaf Extract

The viscous extract obtained was 38.8723 grams as shown in **Figure 1** with a percentage of yield 10.0967%. The yield percentage of previous research was 4.63% [17], so this extraction process is more effective. The drying process at 40°C for three days and the extraction process using 96% ethanol leads to more effective extraction so that the yield is higher. In addition, based on the Pharmacopoeia of Indonesia Herbal Edition II of 2017 states that the percentage of the yield of thick extract of *C. asiatica* herbs should not be less than 7.3%. Thus, the percentage of amendments obtained in this study is good [18]. The percentage of yield can be influenced by the length of the maceration time, the longer the maceration process, the more compounds will be extracted, this can happen because the solvent will be longer in contact with the sample. However, the results of the amendment will tend not to change if they have passed the optimum time limit [19].

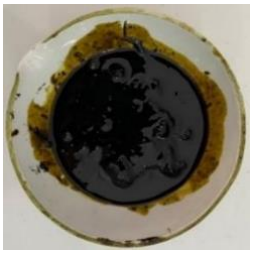


Figure 1. The extract of *Centella asiatica*. L leaves

2.2 Formulation and Evaluation of SNEDDS Preparations of *Centella asiatica* extract

The results of the formulation of the SNEDDS preparation of *C. asiatica* extract can be seen in **Figure 2**. This formulation was chosen based on previous research, but modifications were made to the use of cremophors® RH 40 as surfactants [20]. Cremophor® RH 40 was chosen as a surfactant because it is nonionic which is safer to use compared to ionic surfactants [21, 22]. In addition, chremophor®RH 40 is also more efficient in forming nanoemulsions because they have a high HLB value [23]. Meanwhile, capryol™ 90 as an oil phase has better properties in dissolving drugs that are lipophilic and make them not easily oxidized [24].

Figure 2. SNEDDS *C. asiatica* extract

Based on **Table 1**, it can be seen that the SNEDDS preparation of *C. asiatica* extract has a small particle size. If the particle size is small, the greater the surface area for the absorbed drug will be more effective [25]. The results of the PI test indicate that the SNEDDS preparation of *C. asiatica* extract < 0.7, this value showed good long-term stability because the lower PI value showed that the SNEEDS preparation was more stable because there were fewer aggregating particles. While the value of the polydispersity index is close to zero, the distribution will be even better [26]. In the zeta test, the potential indicates that the SNEDDS preparation of *C. asiatica* extract has good stability when there is a repulsive force between particles that have the same charge when side by side [27]. Based on the percent of transmittance testing, it shows that SNEDDS *C. asiatica* extract forms a clear dispersion in its medium characterized by a percent of transmittance value obtained by

more than 90% [28]. Meanwhile, in the pH test, the results were obtained that the SNEDDS preparation of *C. asiatica* extract was included in the pH criteria that were safe for the skin and could minimize the possibility of irritation [27].

Table 1. Results of the evaluation of SNEDDS preparations of *Centella asiatica* extract

Types of evaluation	Evaluation results	Normal limits
Particle size (nm)	25.2 ± 0.3	≤ 200 nm [20]
Polydisperse index (PI)	0.238 ± 0.024	0.2 – 0.7 [20]
Zeta potential (mV)	-30.6 ± 0.7	< -10 mV [27]
Percent of transmitters(%)	97.036 ± 0.567	> 90% [28]
pH	6.03 ± 0.03	4.5-7.5 [27]

Based on **Table 2**, SNEDDS preparations of *Centella asiatica* extract do not experience instability parameters such as separation, settling, creaming, or cracking. This indicates that all SNEDDS formulas can maintain their stability after centrifugation tests. The centrifugation test is useful to determine the stability of a SNEDDS preparation in resisting the force of gravity after the emulsion is formed. Centrifugation describes the gravitational force that occurs in particles. The small droplet size can minimize brown gravitational and motion forces on particles to prevent phase separation from occurring [29].

Table 2. Results of centrifugation testing of Centrifugation of *Centella asiatica* extract

Separation	Deposition	Creaming	Cracking
-	-	-	-

Based on **Table 3**, it can be seen that the SNEDDS preparation of *C. asiatica* extract does not undergo organoleptic changes such as form, color, and smell. In addition, SNEDDS centella asiatica extract showed can maintain its stability even though it is stored in extreme temperatures [27]. Based on **Table 4**, it can be seen that the SNEDDS preparation of *Centella asiatica* extract has a particle size, PI, and potential zeta whose values are still within the normal range with a particle size of less than 200 nm, a PI value between 0.2 to 0.7 and a potential zeta value of less than – 10 mV [20, 27]. This indicates that SNEDDS pegagan extract can be maintained stability during accelerated stability testing.

Table 3. Test results of freeze-thaw cycle SNEDDS *Centella asiatica* extract for 6 cycles

1st cycle-	Form	Color	Construction	Separation	Deposition	*Creaming*	*Cracking*
0	Thick	Brownish Green	Distinctive	-	-	-	-
1	Thick	Brownish Green	Distinctive	-	-	-	-
2	Thick	Brownish Green	Distinctive	-	-	-	-
3	Thick	Brownish Green	Distinctive	-	-	-	-
4	Thick	Brownish Green	Distinctive	-	-	-	-
5	Thick	Brownish Green	Distinctive	-	-	-	-
6	Thick	Brownish Green	Distinctive	-	-	-	-

Table 4. Results of accelerated stability testing of SNEDDS *Centella asiatica* extract for 4 weeks

Storage	Particle Size (nm)	PI	Zeta Potential (mV)
Week 0	25.2 ± 0.3	0.238 ± 0.024	-30.6 ± 0.7
Week 1	29.2 ± 0.0	0.121 ± 0.050	-12.3 ± 0.6
Week 2	28.5 ± 0.6	0.081± 0.053	-18.0 ± 0.6
Week 3	29.2 ± 1.1	0.195 ± 0.173	-12.1 ± 1.3
Week 4	29.7 ± 0.9	0.161 ± 0.114	-16.5 ± 1.3

2.3 Regeneration of Zebrafish Caudal Fins Test

The results of the calculation of the surface area of the caudal fins of zebrafish in each group can be seen in **Figure 3**. Based on the picture, it can be seen that the surface area of the caudal fin in zebrafish has variations

on the 0 th day (before amputation), this is likely to occur because there is a difference in the size of the caudal fin of the zebrafish and the time the measurement is carried out at a time that is not at the same time. Each group showed slow growth like the normal growth of fish fins every week. Based on the average growth often surface area of fish fins, it can be seen that in the SNEDDS group of *Centella asiatica* extract at a dose of 5 ppm the growth of its fins regenerates faster, that is, the size of the fins in zebrafish is almost close to the size as before amputation on day 14 and shows a greater value of percent regeneration i.e. 81.094%. Meanwhile, in the SNEDDS treatment group, *Centella asiatica* extract at a dose of 10 ppm and 20 ppm, the growth of the fins was slower to regenerate, namely the size of the fins in zebrafish returned to their original size before amputation on day 21, and showed a percent value of regeneration obtained by 54.039% and 56.699%, respectively. This means that the administration of SNEDDS pegagan extract at doses of 10 ppm and 20 ppm did not have a significant effect because the timing of fin growth after amputation was the same as fin growth in the negative control group. In addition, in the solvent control group, there was a fin growth similar to the negative control, namely the fin size in zebrafish was almost close to the size as before amputation on the 21st day with a percent value of regeneration in the solvent control group of 53.738% and negative control of 56.192%. This indicates that solvent control, namely the composition in the SNEDDS used in this study did not affect the growth of caudal fins in zebrafish.

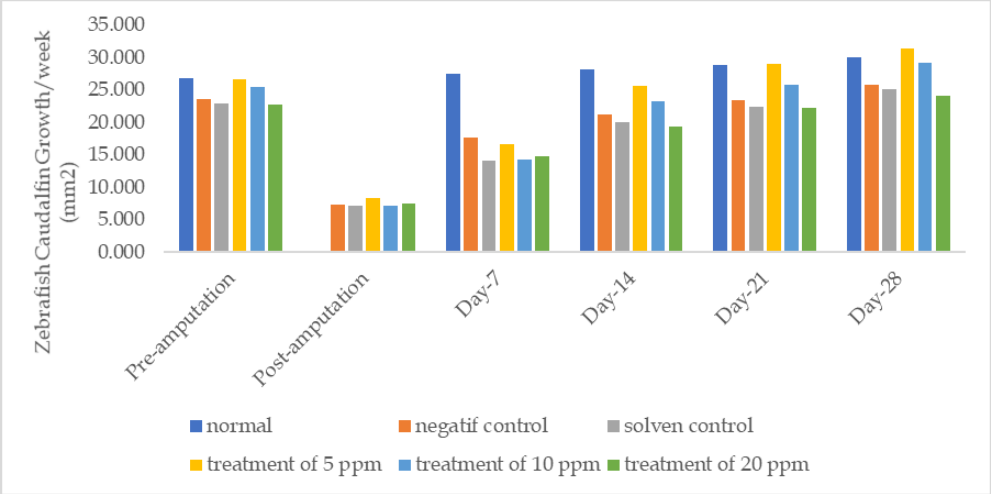


Figure 3. Zebrafish caudal fin area growth chart over 28 days using *Centella asiatica* extract SNEDDS

Based on this, it was seen that after the administration of SNEDDS pegagan extract, zebra fish fins can regenerate during the wound healing process, so in conclusion, asiaticoside contained in *Centella asiatica* can help in inducing the synthesis of type I collagen which plays important role in the formation of skin structure through the activation of TGF-β the I kinase-independent Smad pathway receptor. In addition, some flavonoids have an important role as anti-oxidants in the late stages of wound healing. Furthermore, asiatic acid, which is a group of triterpenoid compounds, is a component that has the best effectiveness in wound healing by inhibiting inflammation, encouraging collagen synthesis, increasing angiogenesis, inducing vasodilation, and reducing oxidative stress in wounds [6].

After the One-Way ANOVA test ($p<0,05$), Post Hoc tests were tested to determine the location of the difference in the average value of the percentage of caudal fins of zebrafish between groups. Based on **Figure 4**, there was a significant difference between the SNEDDS treatment group of 5 ppm dose *C. asiatica* extract SNEDDS and the negative control group, solvent control, SNEDDS treatment group of *Centella asiatica* extract dose 10 and 20 ppm ($p < 0.05$). Thus, it can be concluded that the SNEDDS treatment group of C. asiatica extract at a dose of 5 ppm can regenerate the caudal fins of zebrafish in the wound-healing process.

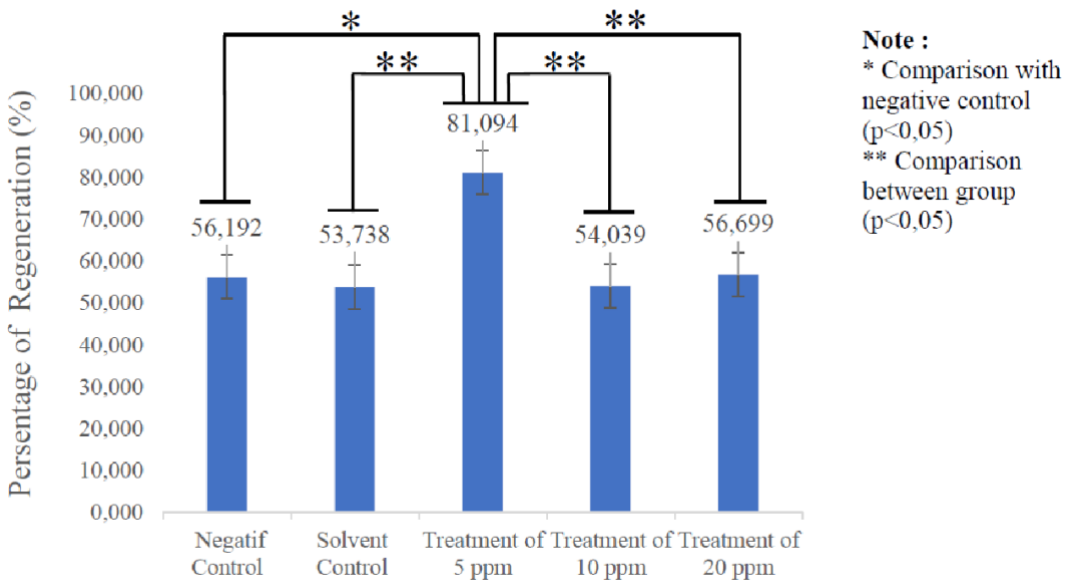


Figure 4. Diagram of the average ratio of the percentage of growth of caudal fins of zebrafish between negative control and solvent control with the SNEDDS group of *Centella asiatica* extract over 28 days

Description: * indicates that there are significant differences between the negative control and solvent control groups; ** indicates that there are significant differences between dose groups.

The zebrafish skin can absorb drugs transdermally depending on the dosage and physicochemical properties of the chemicals contained in the drug, so it can affect the absorption of varying. In addition, the thickness characteristic of the skin layer in zebrafish is only 14 µm in size which can shorten the drug penetration process because zebrafish lack stratum corneum [30]. The physicochemical properties of the chemicals contained in the SNEDDS were the same, so there were no variations in physicochemical properties. The most substantial problems were challenges in controlling and measuring drug exposure. Although it is easy to control the concentration of the drug in the water in the zebrafish aquarium, it will be more difficult to predict how much of the drug the zebrafish will absorb. Some drugs will be absorbed quickly, however, some other drugs will be slow to absorb. Drug levels are usually measured directly in serum or tissue in mammalian animal models, but measurements will be more difficult to do microscopically in zebrafish [31]. In addition, there may be differences in the wound healing process between fish individuals that cause varied growth of fish fins, one of which is the inhibition of signal pathways for wound epithelial formation and inhibition of communication that affects the formation of blastema in the process of regeneration of the caudal fins of zebrafish [32].

This result shows that a SNEDDS pegagan extract dose of 5 ppm has better effectiveness compared to doses of 10 and 20 ppm in regenerating the caudal fins of zebrafish. This phenomenon can occur where an increase in dose does not always correlate with an increase in effect. There is a cut-off value for the amount of drug that has an effect because of possible saturation at the binding site. Under these conditions increasing the dose will not increase the effect.

3. CONCLUSION

Centella asiatica extracts SNEDDS affects the regeneration of the caudal fins of zebrafish in the wound healing process. The results showed that SNEDDS pegagan extract at a dose of 5 ppm has the best effectiveness in regenerating the caudal fins of zebrafish during the wound healing process with a percent regeneration value of 81.094% and a faster fin growth time on day 14.

4. MATERIALS AND METHODS

4.1 Ethical Clearance

This research has received approval from the Ethics Committee for Medical and Health Research, Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta number 4/Ka.Kom. Et/70/KE/IV/2021.

4.2 Materials

The materials used in this study were 6 kg of C. asiatica plants obtained from Kalibawang, Kulonprogo, Yogyakarta, and had been determined from Systematics Laboratory, Faculty of Biology, Gadjah Mada University with certificate number 014963 / S.Tb./II/2021. Tetramin®, tricaine, aqueous, and ethanol 96% feed obtained from the UII Preclinical Test Laboratory, in addition to capryol™90, cremophor® RH, and PEG-400 obtained from the UII Pharmaceutical Technology Laboratory.

Zebrafish obtained from a fish farm in Bogor which was bred at the Preclinical Pharmacology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Islam Indonesia, Yogyakarta. The zebrafish identification process has been carried out at the Indonesian Institute of Sciences (LIPI), Bogor with letter number B-3853 /IPH.1./KS.02.03 / XI / 2017.

The inclusion criteria for the test animals in this study were male and female adult zebrafish measuring 1-1.5 inches, aged 3-6 months with a healthy fish state and no anatomical abnormalities by macroscopically observed. The exclusion criteria for the test animals were fish that were sick, deformed, and died during the study. Before testing, zebrafish will be acclimatized for at least one week in an aquarium with RO (reverse osmosis) water media with ocean salt (0.1%) and equipped with an aerator, water filter, and digital thermometer to monitor the water temperature to be at a temperature of 26^{o}C ± 1 and fed 2 times a day, with an exposure cycle of 14 hours of light and 10 hours of darkness [33].

4.3 Extraction Process

C. asiatica leaves are dried using a cabinet dryer at a temperature of 40^{o}C for 3 days and then pulverized using a grinder. Furthermore, macerated using a 96% ethanol solvent in a ratio of 1: 10 w / v (C. asiatica powder: ethanol) is carried out for 3 days and then re-maceration will be carried out. The powder that has been soaked with ethanol is filtered to obtain a filtrate with the help of a buchner funnel. The filtrate results obtained will be concentrated with the help of a vacuum rotary evaporator (Heidolph) at a temperature of 40^{o}C until it becomes a viscous extract [10]. Percentages of extract yield were calculated with formulae:

Percentage of Extract Yield = (thick extract weight)/(simplicia weight) x 100%

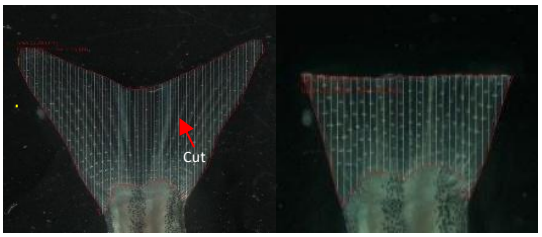
4.4 SNEDDS Formulation and Evaluation

The composition of SNEDDS consists of capryol ™ 90 (3.2 g) as an oil phase, cremophor® RH 40 (3.2 g) as a surfactant, PEG 400 (1.6 g) as a co-surfactant, and a viscous extract of *Centella asiatica* leaves (1.6 g) as an active substance. The thick extract of *Centella asiatica* leaves is weighed first which will then be mixed with capryol ™ 90 and followed by the addition of cremophor® RH 40 and PEG 400 and then ultrasonicated (Biologics Model 300 V /T) until homogeneous.

Evaluation of SNEDDS preparations was carried out in the form of particle size tests, Polydispersity Index (PI), and potential zeta measured using the Particle Size Analyzer tool (Horiba SZ 100Z) before the test SNEDDS diluted 100x. The percentage of transmitters was measured using a UV-Vis (Hitachi) spectrophotometer with a wavelength of 650 nm and aqueous as blanks. pH testing using a pH meter tool (Horiba). Thermodynamic stability tests are carried out in two stages: centrifugation and Freeze-thaw cycle tests. The centrifugation test was performed at a speed of 2500 rpm for 40 minutes (Nuve NF 400). Then the Freeze-thaw cycle test was carried out by storing SNEDDS at a temperature of 4^{o} ± 2^{o}C and then transferring them to a temperature of 25^{o} ± 2^{o}C, the storage process at each temperature is 24 hours (one cycle). The test was carried out over six cycles and it was seen whether there were organoleptic changes and phase separation, settling, creaming, or cracking occurred. Finally, accelerated stability testing was carried out, namely changes in particle size, PI, and potential zeta using PSA on SNEDDS stored in climatic chambers with storage of 40^{o}C ± 2^{o}C/75% RH ± 5% RH. Furthermore, SNEDDS was observed every week for 4 weeks [23, 27, 34, 35]. In the future, it is necessary to carry out another stability test to ensure the stability of the SNEDDS *C. asiatica* extract using the accelerated stability test method for 12 weeks.

4.5 Zebrafish Caudal Fins Regeneration Test

Zebrafish were given anesthesia using tricaine by immersion at a concentration of 0.168 mg /ml [36]. The documentation was carried out in the negative control group, solvents, treatment of 5 ppm, 10 ppm, and 20 ppm to determine the state of the fins of zebrafish before amputation was carried out. Furthermore, the caudal fin is cut straight using a bisturi on one segment of the fin located under the first branching or cut in half the length of the tail fin of the fish vertically as shown in **Figure 5**. After the cutting of the caudal fin is observed again under a microscope and carried out documentation is considered the state of the fin 0 hours after cutting. The area of the caudal fin was calculated using the Zeiss zen blue program which is the application of the microscope used. There is a camera in the microscope (axiocam 208) which helps mark the area to be calculated.



(a) (b)

Figure 5. Cutting zebrafish caudal fins

(a) Before the cutting of the fins; (b) After cutting of the caudal fins

Amputated zebrafish are transferred into the aquarium based on their test group. The test group was divided into 6 groups consisting of 8 fish each (**Table 5**). Furthermore, observations were made of the regeneration of the caudal fins of zebrafish using stereo microscopes (Zeiss) on the 7th, 14th, 21st, and 28th days. Then the calculation of the fin regeneration area is carried out to find out the results of fin growth. The calculation method is carried out as follows:

Missing area = Area before amputation – Post-amputation area

Regeneration area = Area of the nth day post-amputation – Missing area

Regeneration percentage = = $\frac{Regeneration\ area}{Missing\ area}$ x 100%

After that, statistical analysis was carried out using the one-way ANOVA method to determine the difference in the growth of fish caudal fins in each test group. The test results can be considered statistically meaningful if the $p<0.05$ value.

Table 5. The group's test of wound-healing activity of *Centella asiatica* L leaf extract SNEDDS in zebrafish

Group	Treatment
Control normal	Not amputated and not given treatment, only fed and drank
Control negative	Amputated and not given SNEDDS *Centella asiatica* extract
Solvent control	Amputated and given SNEDDS without *Centella asiatica* extract
Treatment 1	Amputated and administered SNEDDS *Centella asiatica* extract dose of 5 ppm
Treatment 2	Amputated and administered SNEDDS *Centella asiatica* extract dose of 10 ppm
Treatment 3	Amputated and given SNEDDS *Centella asiatica* extract dose of 20 ppm

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Author contributions: Concept – F.H., L.C; Design – F.H, T.S., LC.; Supervision – F.H., L.C;.; Resources – F.H, T.S., LC Materials – F.H.; Data Collection and/or Processing – T.S; Analysis and/or Interpretation – F.H, T.S., LC.; Literature Search –T.S; M.H; Writing – T.S; M.H..; Critical Reviews – F.H; M.H.; L.C.

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