



Wound healing activity of *Centella asiatica* extract via inflammatory gene regulation in zebrafish (*Danio rerio*)

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Background

Centella asiatica (L.) Urb has been recognized for its potential to accelerate wound healing and support tissue repair and regeneration. This effect may involve the regulation of pro-inflammatory genes such as *Interleukin-1 Beta* (*IL-1β*) and *Matrix Metalloproteinase-9* (*MMP-9*).

Objective

This study aimed to evaluate the wound healing potential of ethanolic *C. asiatica* leaf extract by assessing caudal fin regeneration and the expression levels of *IL-1β* and *MMP-9* in zebrafish (*Danio rerio*).

Materials and methods

Zebrafish were assigned to control, 7.5-ppm extract, and 10-ppm extract groups ($n = 20$ per group) and observed for 7 days post-caudal fin amputation. The caudal fin regeneration was quantified by means of a stereo microscope, and inflammatory gene expression (*IL-1β* and *MMP-9*) was analyzed using qRT-PCR.

Results and conclusion

The average percentage of caudal fin regeneration was 40.51% in the normal group, 46.32% in the 7.5-ppm extract group, and 47.59% in the 10-ppm extract group. A significant reduction in gene expressions was observed in the 10-ppm group, with $-1.097x$ in *IL-1β* expression and $-0.276x$ in *MMP-9* expression compared to the control.

These findings suggest that *C. asiatica* extract enhances wound healing in zebrafish by downregulating pro-inflammatory genes.

Keywords

Caudal fin, *Centella asiatica*, wound healing, *IL-1β*, *MMP-9*

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Introduction

Empirical evidence shows that *Centella asiatica* (Linn.) Urban, commonly known as pegagan, is widely used in Indonesia as an alternative therapeutic agent. It contains a variety of bioactive compounds, including alkaloids, triterpenoids/steroids, flavonoids, tannins, and saponins, which are known to possess antioxidant, anti-inflammatory, and antimicrobial properties [1, 2]. Asiaticoside is a triterpenoid compound consisting of a polar glycone moiety and a non-polar aglycone moiety. Asiaticoside has been reported to promote wound healing and is effective in supporting tissue repair and regeneration in animal models [3]. Asiaticoside particularly exhibits significant anti-inflammatory effects. Studies using standardized *C. asiatica* extract have shown that it can reduce erythema, support re-epithelialization, and improve tissue texture by enhancing collagen production and optimizing the wound healing process [4].

An animal model commonly used in wound healing studies is the zebrafish (*Danio rerio*) through the observation of caudal fin regeneration. This model is advantageous due to its high genetic similarity to humans, with more than 70% of the protein-coding genes being homologous,

and because the species is capable of regenerating tissues after amputation, the procedure is non-lethal, and the regeneration process is relatively fast and easily observable [5]. Previous research has shown that *C. asiatica* leaf extract contributes to the acceleration of incisional wound healing in rats by enhancing the wound closure process [6]. It has been reported that the ethanolic extract of *C. asiatica* leaves promotes the regeneration of amputated zebrafish caudal fins and upregulates the expression of the genes associated with the wound healing process [7].

Wound healing in zebrafish involves the regulation of multiple genes, including those expressed during the inflammatory phase, such as *interleukin-1β* (*IL-1β*) and *Matrix Metalloproteinase-9* (*MMP-9*). Purified asiaticoside (with 90% purity) has been shown to significantly reduce *IL-1β* transcription, suppress *IL-6* gene expression, and inhibit the upregulation of *Tumor Necrosis Factor -Alpha* (*TNF-α*) transcription [8]. Meanwhile, the *MMP-9* gene plays a critical role in wound healing. Its expression is upregulated during the early stages of the healing process, although such increase is transient [9, 10]. The upregulation of *MMP-9* is stimulated by pro-inflammatory cytokines at the site of inflammation. *Matrix Metalloproteinase-9* is secreted by macrophages and fibroblasts, and it functions

in extracellular matrix remodeling, keratinocyte migration, and vertebrate tissue regeneration [11, 12].

Observations of *IL-1 β* gene expression in the caudal fin blastema of zebrafish can be conducted through the quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) method. This technique offers high sensitivity, a wide dynamic range, and a low risk of cross-contamination compared to other molecular methods [13]. In studies of wound healing mechanisms at the molecular level, zebrafish serve as a well-established model organism due to its genetic similarity to humans, including the *MMP-9* gene, which plays an essential role in vertebrate regeneration. Notably, *MMP-9* expression is localized at the distal tip of the regenerating caudal fin blastema [12]. The caudal fin of zebrafish is considered an ideal tissue for regeneration studies as it is the largest fin, has a simple anatomical structure, and undergoes rapid regeneration [14]. In addition, qRT-PCR is recognized as a sensitive and accurate method for quantifying gene expression through the amplification of target deoxyribonucleic acid (DNA) sequences [15]. These advantages support the use of zebrafish and qRT-PCR as a reliable system to assess the impact of the ethanolic extract of *C. asiatica* on the expression of the genes involved in the wound healing process, particularly *IL-1 β* and *MMP-9*.

Materials and methods

Ethical clearance

This study has obtained an ethical clearance certificate from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta, with the reference number 2/Ka.Kom.Et/70/KE/III/2023 and 6/Ka.Kom.Et/70/KE/III/2023.

Materials

The equipment used in this study included an aquarium, qRT-PCR machine (Bio-Rad, USA), stirring rod, cabinet dryer (CANADERA, Indonesia), Buchner funnel (GAST Group Ltd, Shanghai-China), grinder (ZT-300 FOMAC, Indonesia), micropipettes (Fisherbrand, USA), stereo microscope (ZEISS Discovery.V8, Germany), glassware (IWAKI, Japan), fish maintenance equipment (Tetra, Germany), sterile 1.5 mL and 200 μ L blue microcentrifuge tubes (Star Dec, China), sterile PCR tubes (Biologix, USA), scalpel blade no. 10 (GEA, Indonesia), centrifuge (DIAB D2012plus, China), vacuum rotary evaporator (Heidolph, Korea), vortex mixer (Biosan, Latvia), water bath (Memmert, Germany), XTerra® Prep MS C18 5 μ L column sized 7,8 x 150 mm (Waters Corporation, USA), Photodiode Array Detector (Waters 2998, Waters Corporation, USA).

The materials used in this study included Reverse Osmosis (RO) water, bidistilled water, distilled water, amyl alcohol (Merck, Germany), Aqua Safe (Tetra, Germany), β -mercaptoethanol (SIGMA, USA), 70% ethanol (Merck, Germany), 96% ethanol p.a. (pro analysis), cDNA synthesis kit (ThermoFisher, USA), Tissue Total RNA Mini Kit 050 (Geneaid, Taiwan), PCR Master Mix (SmoBio, Taiwan), artificial sea salt (ASW), micro pellet fish feed (300–400 μ m, Hikari, Japan), forward and reverse primers for *β -actin*, *IL-1 β* , and *MMP-9*, sterile blue tips and yellow tips (Extra Gene, Taiwan), tricaine (Sigma-Aldrich, USA).

Preparation of the ethanolic extract of pegagan (*C. asiatica*) leaves

Pegagan leaves (*Centella asiatica* (L.) Urb.) were collected from Kalibawang, Kulon Progo, Yogyakarta, Indonesia. The preparation of pegagan leaf extract was carried out at the Pharmaceutical Biology Laboratory, Universitas Islam Indonesia. The pegagan leaves were sorted and cleaned, then dried by using a cabinet dryer at a temperature of 35–40°C for 2 days [16]. Once dried, the leaves were ground using a grinder to obtain powder.

The extraction process of pegagan leaves was conducted through the maceration method with 96% ethanol as the solvent. A total of 500 grams of pegagan powder was soaked in 5 liters of 96% ethanol with a ratio of 1:10 (b/v) for 72 hours. Occasional stirring was performed during the extraction process. After the maceration was completed, the mixture was filtered using a Büchner funnel, and the remaining residue was remacerated twice. The resulting liquid extract was then concentrated using a vacuum rotary evaporator at 40°C to obtain a thick pegagan extract [16].

Animal treatment procedures

The inclusion criteria for the experimental animals in this study were healthy adult zebrafish aged 3–6 months. Healthy fish were identified based on the swimming activity typically indicated by the fish staying in the middle of the aquarium, being active (not motionless), and showing no signs of infection or inflammation, such as skin lesions, raised scales, swollen abdomen, or skin redness. The exclusion criteria included the fish being sick, deformed, or dead during the study. The zebrafish were maintained in glass aquariums equipped with an aerator, a water filter, and a digital thermometer. An acclimatization period was conducted for two weeks prior to the experiment. This study used three experimental groups ($n = 20$ fish per group), a normal control group, a 7.5-ppm extract group, and a 10-ppm extract group. All the zebrafish in each experimental group underwent caudal fin amputation. Prior to the procedure, the fish were anesthetized using a tricaine solution. Observation of the caudal fin regeneration was conducted on the 7th day after amputation.

Gene expression analysis using Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

From each test group, blastema samples were collected on day 7 post-amputation. The samples were then stored in a freezer at -80°C. For each treatment group, 25 mg of the collected blastema sample was used for RNA isolation by using the Tissue Total RNA mini kit 050 (Geneaid, Taiwan). The isolated RNA samples were then used for cDNA synthesis using a cDNA synthesis kit (ThermoFisher, USA). The analysis of *MMP-9* and *IL-1 β* gene expressions was performed by using qRT-PCR following a modified protocol based on previous studies and utilizing the same primer sequences shown in Table 1 [17, 18]. The qRT-PCR cycle protocol was carried out by using the SmoBio Excel Taq 2X Fast Q-PCR Master Mix. The PCR cycle was set for 40 cycles with an initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, and annealing at 58.2°C for 1 minute. The results obtained were amplification curves and Ct values indicating gene amplification.

Table 1 Primer sequences for β -actin, *IL-1 β* , and *MMP-9* genes

Gene	Sequence	Accession number
β -actin	F TTCACCACCACAGCCGAAAGA	AF057040
	R TACCGCAAGATTCCATACCCA	
<i>IL-1β</i>	F TCAAACCCCAATCCACAGAG	NM212844
	R TCACTTCACGCTCTTGATG	
<i>MMP-9</i>	F TTTGCCCTGATCGTGATAC	AY151254
	R GGGAAACCCTCCACGTATT	

*Primer sequences were adopted and modified from previous study [18] and validated using the NCBI Primer-BLAST tool.

The β -actin gene was used as a housekeeping gene to normalize gene expression levels. Following normalization, the relative expression of target genes (*MMP-9* and *IL-1 β*) was determined using the Livak method ($2^{-\Delta\Delta Ct}$) to calculate fold changes. The $\Delta\Delta Ct$ was then determined by comparing the ΔCt of each experimental group to that of the normal group, which served as the calibrator. The normal group represented the untreated and uninjured condition and thus served as the reference baseline for evaluating relative gene expression across all treatment groups. Fold changes in gene expression were calculated accordingly.

Statistical analysis

The percentage of regeneration data was statistically analyzed by using the One-Way ANOVA in IBM SPSS version 29.0.1.0 to determine the significance of differences among the groups ($p < 0.05$).

Results and discussions

Extraction of *C. asiatica* leaves

The *C. asiatica* leaves were extracted through maceration with 96% ethanol, resulting in a thick extract with dark green color, specific odor, and bitter taste, in accordance with the Indonesian Herbal Pharmacopeia [19]. A total of 54.97 grams of extract was obtained with a yield of 10.99% (w/w), which exceeds the minimum requirement of 7.3% (w/w). This yield is also higher than that reported in a previous study (9.41%), possibly due to differences in the temperature, stirring, and soaking duration during the extraction process [3].

The ethanolic extract of *C. asiatica* leaves was subjected to phytochemical screening and asiaticoside identification. The extract contains alkaloids, flavonoids, saponins, triterpenoids, and tannins. These results are consistent with previous studies reporting similar phytochemical profiles in *C. asiatica* leaves [16, 20].

The identification of asiaticoside in the *C. asiatica* extract was carried out using High Performance Liquid Chromatography with a C18 column and a gradient system of water-acetonitrile. The chromatogram shows a retention time of 16.173 minutes for the asiaticoside standard (Fig. 1a) and 16.076 minutes for the extract (Fig. 1b), indicating the presence of asiaticoside. The asiaticoside content in the extract was 9.07%, thus exceeding the minimum requirement of 0.90% as stated in the Indonesian Herbal Pharmacopeia [19]. This value is significantly higher than that reported in another study (0.01%), possibly due to differences in the plant location, harvesting time, and solvent used [3].

Effect of *C. asiatica* leaf extract on zebrafish caudal fin regeneration

The average percentage of caudal fin regeneration in zebrafish over a 7-day period showed that the *C. asiatica* ethanol extract influenced the regeneration process. The highest regeneration rate was observed in the 10-ppm extract (47.59%), followed by the 7.5-ppm extract and the normal group, as shown in Fig. 2. The normality (Shapiro-Wilk) and homogeneity tests indicated that the data were normally distributed and homogeneous ($p > 0.05$). The one-way ANOVA analysis demonstrated a statistically significant difference between groups ($p < 0.05$), followed by the Tukey's Post Hoc test, which revealed significant differences between the normal group and both extract-treated groups. However, no significant difference was found between the 7.5-ppm and 10-ppm extract groups.

These findings indicate that *C. asiatica* extract substantially accelerates caudal fin regeneration, although the lack of a significant difference between the 7.5-ppm and 10-ppm groups suggests a physiological plateau. This saturation point, occurring at approximately 10 ppm, implies that the maximum threshold for receptor activation or signaling pathway stimulation has been achieved. Beyond this concentration, additional extract does not yield proportional increases in tissue outgrowth, a phenomenon often observed in herbal-based pharmacological interventions [21].

The efficacy of the extract in this study was further optimized by the utilization of zebrafish with consistent size and age, ensuring uniform transdermal drug absorption. In this model, the local effect on fin regeneration is highly dependent on the physicochemical properties of the tested compounds and their ability to penetrate the skin. Although the skin structure of zebrafish, comprising the epidermis, dermis, and hypodermis, is comparable to human skin, notable differences facilitate faster compound delivery. Specifically, the average skin thickness of an adult zebrafish is only approximately 14 μ m, and the reduced or absent stratum corneum allows for more rapid chemical penetration compared to adult human skin [22]. This enhanced permeability allows bioactive compounds, particularly triterpenoids, to reach the target site more efficiently and initiate tissue repair.

The observed wound healing activity is primarily associated with the presence of major triterpenoid compounds, including asiatic acid, madecassic acid, asiaticoside, and madecassoside. Asiaticoside, in particular, has been reported to stimulate type I collagen synthesis in dermal fibroblasts and elevate antioxidant levels at the wound site [7]. These

antioxidant effects play an important role in reducing oxidative stress, thereby facilitating progression through the key stages of regeneration, including inflammation, proliferation (blastema formation), and remodeling. Caudal fin regeneration is a highly orchestrated event requiring a precise transition between these phases [14]. The *C. asiatica* extract appears to act as a biochemical bridge, modulating the environment at the amputation site to ensure that tissue synthesis occurs without the delays typically induced by physiological stress or unbalanced signaling.

During the early post-amputation phase, a transient inflammatory response is indispensable for clearing necrotic debris and triggering repair signals. Nevertheless, chronic or excessive inflammation is known to inhibit the reprogramming of cells into a regenerative state. *Centella asiatica* is well-documented for its potent anti-inflammatory properties, primarily through the inhibition of pro-inflammatory cytokines such as TNF- α and IL-1 β

[23]. In this study, the extract likely maintained a balanced inflammatory response, preventing excessive stress and enabling progression into the proliferative phase, which is critical for successful regeneration [23].

The proliferative phase in zebrafish is characterized by the development of the blastema, a mass of undifferentiated mesenchymal cells that serves as the engine for tissue regrowth. The increased regeneration percentage in the extract-treated groups suggests that *C. asiatica* facilitates blastema formation and stimulates cellular proliferation. Bioactive triterpenes, such as asiaticoside found in the extract, have been shown to upregulate the Wnt (Wingless-related integration site)/ β -catenin signaling pathway, which is essential for the maintenance and expansion of the blastema cell population [24]. This molecular stimulation leads to the accelerated elongation of the fin rays and intersegmental tissue observed in our 7-day observation period.

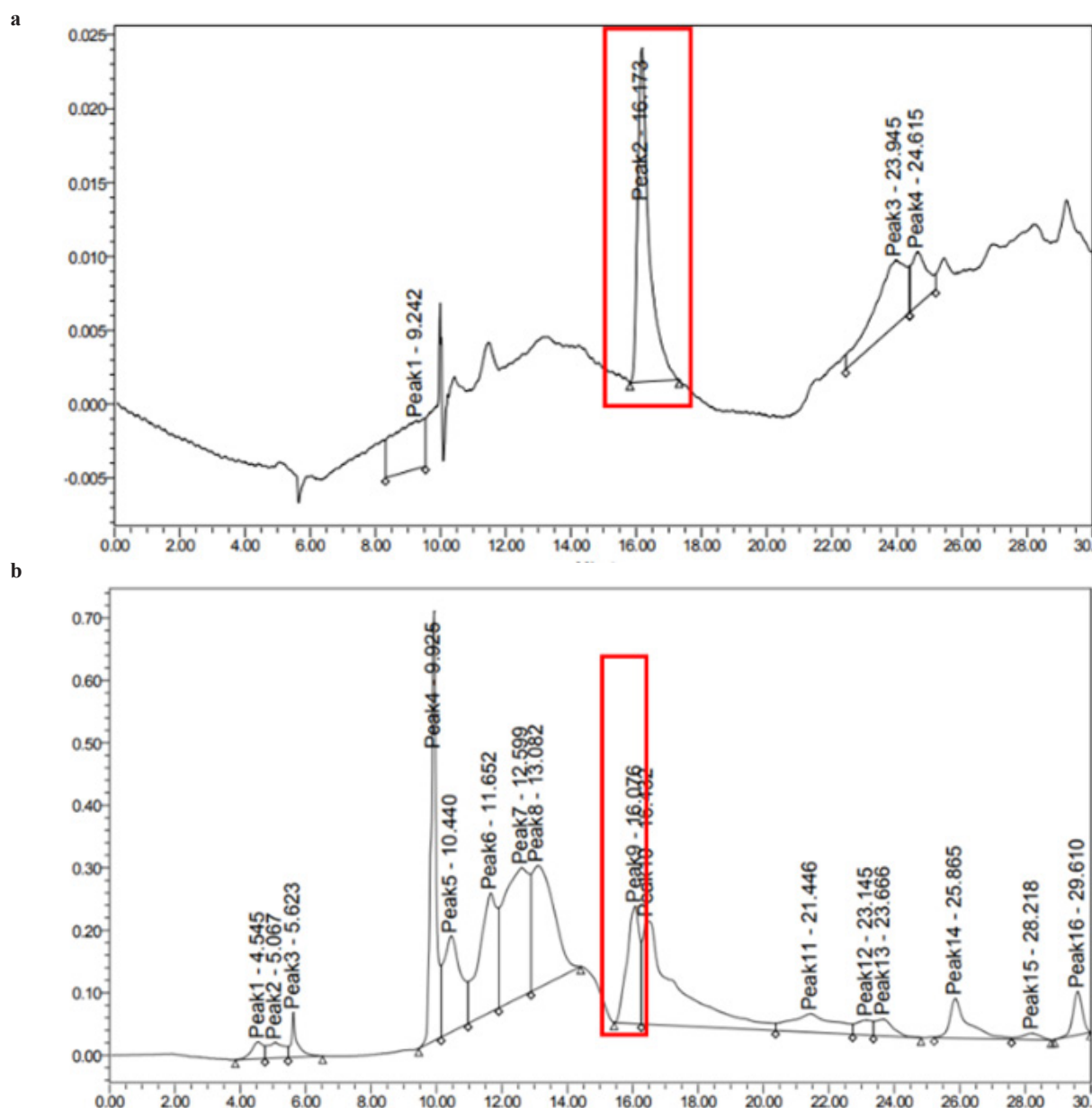


Fig. 1 Chromatograms of asiaticoside in (a) the standard solution and (b) the ethanolic extract of *C. asiatica* leaves [Description: The peak highlighted with a red box indicates the presence of asiaticoside. Chromatographic conditions. Mobile phase: gradient elution from 100% water to 100% acetonitrile; stationary phase: Xterra® Prep MS C18 column (5 μ m, 7.8 $\times$ 150 mm)]

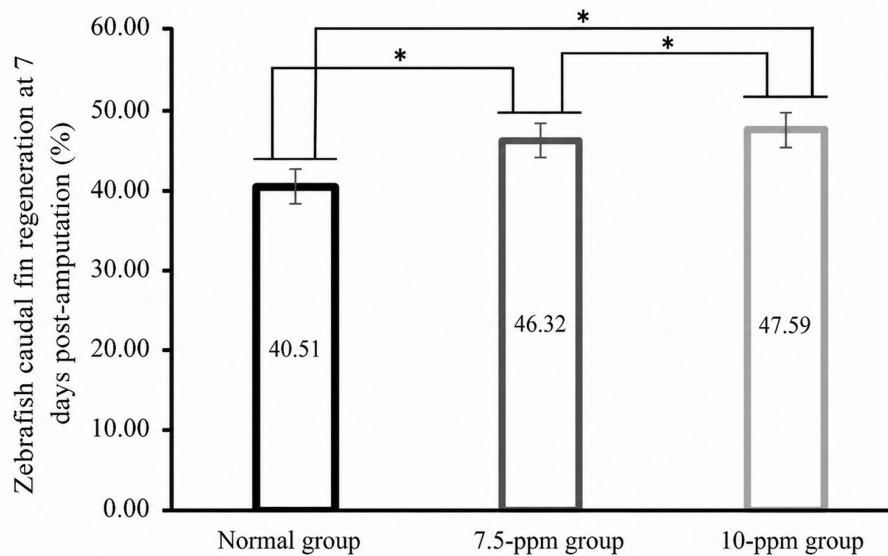


Fig. 2 Mean percentage of caudal fin regeneration in zebrafish in 7 days post-amputation [Statistical analysis using ANOVA followed by Tukey's post-hoc test. The * sign indicates significance ($p < 0.05$)]

In the final stage of wound healing, the remodeling phase is characterized by the maturation of newly formed tissue and the restoration of its structural integrity. *Centella asiatica* has been extensively reported to promote type I collagen synthesis and support extracellular matrix (ECM) stabilization, contributing to improved tissue organization [25]. Proper remodeling ensures that the regenerated fin develops into a functional structure rather than merely an accumulation of tissue. These findings are in agreement with previous studies, Nurkhasanah et al. (2024) reported that *C. asiatica* enhances caudal fin regeneration, as reflected by the upregulation of genes associated with tissue growth and repair, including *sonic hedgehog (shh)*, *insulin-like growth factor 2a (igf2a)*, *bone morphogenetic protein 2b (bmp2b)*, and *collagen type 1 alpha 2 (coll1a2)* [7]. In addition, a study by Hayati et al. (2022) demonstrated that *C. asiatica* extract, both in its crude form and when formulated as a self-nanoemulsifying drug delivery system (SNEDDS), significantly improved caudal fin regeneration across various concentrations [26].

Observation results of *IL-1 β* and *MMP-9* gene expression on caudal fin regeneration in zebrafish

The analysis of *IL-1 β* and *MMP-9* gene expressions in zebrafish caudal fin regeneration was performed by using the qRT-PCR technique, following a protocol modified based on the kit brochure and initial optimization of the annealing temperature obtained at the beginning of the study. Identification using RT-PCR yields Ct (cycle threshold) values, which are defined as the number of amplification cycles required to reach a threshold. A high Ct value indicates a low expression of the target gene [21]. The gene expressions of *IL-1 β* and *MMP-9* during wound healing in zebrafish are presented in Table 2.

Observation of *IL-1 β* and *MMP-9* gene expressions revealed that the group treated with 10-ppm ethanol extract of *C. asiatica* exhibited the lowest gene expression levels compared to the other groups. This finding was consistent with the Ct values obtained, where the 10-ppm group showed higher Ct values, indicating lower target gene copy numbers. As shown in Fig. 3, the most significant reduction in gene expression

occurred in the 10-ppm treatment group, with *IL-1 β* expression decreased by -1.097 -fold and *MMP-9* by -0.276 -fold relative to the normal group. These results suggest that the 10-ppm extract of *C. asiatica* effectively suppresses pro-inflammatory gene expressions associated with the wound healing process.

Based on the results of this study, the expression pattern of the *IL-1 β* gene was consistent with the caudal fin regeneration outcomes in zebrafish. The group treated with 10-ppm ethanol extract of *C. asiatica* demonstrated the most significant reduction in *IL-1 β* expression, accompanied by a higher percentage of tissue regeneration compared to the normal group. Although no statistically significant difference was observed between the 10-ppm and 7.5-ppm extract groups, the trend still indicated a dose-dependent decrease in gene expression and an increase in regenerative ability. This suggests that *C. asiatica* extract, particularly at a dose of 10-ppm, effectively modulates the inflammatory response as indicated by reduced *IL-1 β* levels. These findings are consistent with a study by Chen et al. (2014), which reports a significant downregulation of *IL-1 β* transcription in an ischemic mouse model following treatment with *C. asiatica*, attributed to its anti-inflammatory properties [8].

In addition to *IL-1 β* , the expression of the *MMP-9* gene also demonstrated a significant reduction in both the 7.5-ppm and 10-ppm treatment groups compared to the normal control. This suggests that *C. asiatica* extract may contribute to the suppression of *MMP-9* activity, a matrix metalloproteinase involved in extracellular matrix remodeling and re-epithelialization during wound healing [12]. The observed decrease in *MMP-9* expression may reflect a faster transition from the inflammatory phase to the proliferative phase of tissue regeneration. Similar findings are reported by Edirisinghe et al. (2020), who demonstrate that *MMP-9* expression increases significantly on the first day post-injury in wounds treated with *Spirulina maxima*-based pectin, followed by a notable decline by day 7, coinciding with the onset of healing [22].

Table 2 Gene expressions of *IL-1β* and *MMP-9* during wound healing in zebrafish 7 days post-amputation following the administration of *C. asiatica* ethanol extract

Gene	Treatment group	Average gene expression ± SD*
<i>IL-1β</i>	Normal Group	1.08 ± 0.54
	7.5-ppm Extract Group	0.71 ± 0.08
	10-ppm Extract Group	0.08 ± 0.01
<i>MMP-9</i>	Normal Group	2.48 ± 3,16
	7.5-ppm Extract Group	1.74 ± 1.58
	10-ppm Extract Group	0.53 ± 0.54

*Gene expression values were calculated using the Livak method ($2^{-\Delta\Delta C_t}$) for each group with three replicates

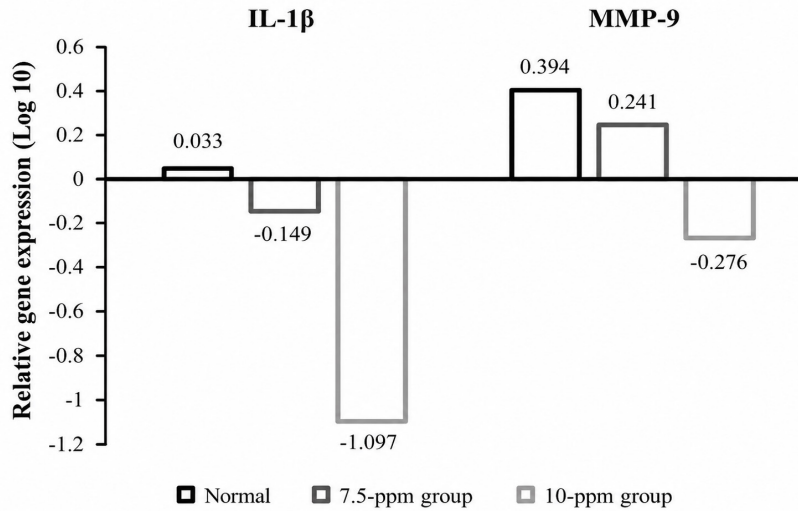


Fig. 3. Comparative of average relative gene expression values (log₁₀ based)

In contrast, untreated wounds demonstrate sustained *MMP-9* expression during the early post-injury period (1-14 days), with a delayed decline observed in the later stages [23]. The *MMP-9* expression is heavily influenced by the presence of pro-inflammatory cytokines, such as *IL-1β* and *TNF-α*, which stimulate *MMP* gene transcription. Therefore, the reduction in *IL-1β* expression observed in this study may have contributed indirectly to the suppression of *MMP-9* expression [24, 25].

Asiaticoside as a key bioactive compound in *C. asiatica*, is known for its ability to downregulate pro-inflammatory cytokines and support the wound healing process. Its modulation of *MMP-9* expression is believed to occur through enhancement of re-epithelialization by promoting the degradation of the basement membrane, thus allowing keratinocyte migration and wound closure [4]. Physiologically, *MMP-9* expression typically increases following tissue injury; however, prolonged overexpression may delay wound healing and contribute to chronic wounds [23]. Therefore, the lower *MMP-9* expression observed in the treatment groups may serve as an indicator of more efficient healing progression [26].

A limitation of this study is that the gene expression and caudal fin regeneration were only assessed at a single time point of day 7 post-amputation. As a result, the dynamic changes in gene expressions, such as the peak levels and the return to baseline during the resolution phase, were not captured. Furthermore, this study focused solely on two genes (*IL-1β* and *MMP-9*), whereas the wound healing process involves a complex interplay of numerous genes

and signaling pathways, including *TNF-α*, *TGF-β*, *VEGF*, and *IL-10*, which may also influence *MMP-9* activity and tissue repair [27].

Conclusion

The administration of the ethanol extract of *C. asiatica* was shown to enhance caudal fin regeneration in zebrafish and reduce the expression of *IL-1β* and *MMP-9* pro-inflammatory genes. The 10-ppm extract group exhibited the highest regeneration rate and the most significant reduction in gene expression compared to the other groups. These findings indicate that *C. asiatica* has the potential as a natural therapeutic agent for promoting wound healing through the modulation of inflammation and stimulation of tissue regeneration.

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Conflict of interest

The authors declare there are no conflicts of interest.

Author’s contributions

A.A and A.D.K conducted experimental work, collected the data, and drafted the manuscript. L.N contributed to

data collection and assisted in manuscript writing. F.H and R.I supervised the study, provided methodological and analytical guidance, and revised the manuscript. M.H.M.N contributed to manuscript writing and provided critical feedback on research design and interpretation of results. All authors reviewed and approved the final manuscript.

Ethical considerations

This study has obtained an ethical clearance certificate from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta, with the reference number 2/Ka.Kom.Et/70/KE/III/2023 and 6/Ka.Kom.Et/70/KE/III/2023.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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