Original Research Article

Nanoparticle-Assisted Enhancement of Therapeutic Compounds in *Centella asiatica*: A Qualitative Analysis

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ABSTRACT

Centella asiatica (CA) is renowned for its medicinal properties, attributed primarily to its triterpenoid saponins: asiaticoside, asiatic acid, madecassoside, and madecassic acid. These compounds support collagen synthesis, wound healing, and exhibit antioxidant and anti-inflammatory properties. However, natural CA cultivation yields insufficient bioactive compounds to meet market demands. This study investigates zinc oxide nanoparticles (ZnONPs) as a means to enhance their production. ZnONPs were applied in varying concentrations (50, 100, 150, 200, and 250 mg/L) to hydroponically grown CA. Plants were harvested at intervals of 7, 14, 21, and 28 days, and analyzed using high-performance liquid chromatography (HPLC). ZnONPs significantly increased asiaticoside and madecassoside levels, particularly at 100 and 150 mg/L within 7 to 14 days. Asiaticoside peaked at 100 mg/L after 14 days, while madecassoside reached maximum levels at 150 mg/L ZnONPs concentration. Both compounds showed significant increases during the early stages of ZnONPs. Asiatic acid and madecassic acid also showed increased production, though the enhancement was less detected compared to asiaticoside and madecassoside. Asiatic acid exhibited significant peaks at 150 mg/L and 200 mg/L, while madecassic acid showed moderate enhancement, with peaks at 100 mg/L and 150 mg/L ZnONP concentrations. However, prolonged exposure and higher concentrations of ZnONPs led to a plateau or decline in compound production, likely due to nanoparticle toxicity. This study highlights the potential of nanotechnology in agriculture to sustainably enhance the production of valuable secondary metabolites in medicinal plants, meeting both pharmaceutical and cosmetic industry needs for consistent and high-quality bioactive compounds.

Keywords: *Centella asiatica*, zinc oxide nanoparticle, asiaticoside, madecassoside

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1.0 Introduction

Centella asiatica (CA), also known as *pegaga* is a perennial herbaceous plant belonging to the Apiaceae family (1). It has a rich history of use in traditional medicine systems, such as Ayurveda, Unani, and Traditional Chinese Medicine, where it is revered for its therapeutic properties (2). For centuries, CA has been utilized for wound healing, treatment of skin diseases, and enhancing cognitive functions (3). The plant is indigenous to the wetlands of Southeast Asia but is now cultivated in various parts of the world due to its medicinal value (4).The medicinal properties of CA are largely attributed to its diverse array of bioactive compounds. Key among these are triterpenoid saponins, particularly asiaticoside, Asiatic acid, madecassoside, and madecassic acid (5). Asiaticoside and madecassoside are glycosides known for their ability to promote collagen synthesis and wound healing, making them valuable in dermatology and cosmetics (6). Asiatic acid and madecassic acid, the aglycones of asiaticoside and madecassoside, exhibit strong antioxidant and anti-inflammatory properties, which are beneficial in treating various skin conditions and preventing oxidative stress-related disorders (7). Despite its therapeutic potential, the naturally grown CA produces insufficient quantities of these bioactive compounds to meet the escalating market demand. Factors such as environmental conditions, soil quality, nutrition content and plant maturity significantly influence the concentration of these compounds, resulting in variability and inconsistency in their availability (8, 9). This poses a substantial challenge for the pharmaceutical and cosmetic industries, which require a steady and standardized supply of these bioactive compounds for product formulation. The increasing consumer preference for natural and organic products further exacerbates the demand-supply gap. There is a growing awareness of the side effects associated with synthetic chemicals, driving consumers towards natural remedies (10). Consequently, there is an urgent need for innovative and sustainable methods to enhance the production of bioactive compounds in CA to meet the market demand.

Nanotechnology has emerged as a revolutionary approach in various fields, including agriculture. The application of nanoparticles in agriculture has shown promising results in enhancing plant growth, nutrient uptake, and stress resistance (11, 12). Metal and metal oxide nanoparticles, such as zinc oxide nanoparticles (ZnONPs), have garnered significant attention due to their unique physicochemical properties. ZnONPs are known for their biocompatibility, low toxicity, and ability to improve plant metabolic processes (13). As zinc is a necessary micronutrient in plants, these nanoparticles can enhance photosynthetic efficiency, increase water and nutrient uptake, and induce the production of reactive oxygen species (ROS) that activate defense-related secondary metabolite pathways (14-16). These properties make ZnONPs an attractive candidate for enhancing the biosynthesis of valuable secondary metabolites in medicinal plants.

The primary objective of this study was to enhance the production of four key bioactive compounds—asiaticoside, asiatic acid, madecassoside, and madecassic acid—in CA using ZnONPs. By leveraging the unique properties of ZnONPs, the study aimed to address the challenge of insufficient bioactive compound production in naturally grown CA. Five different concentrations of ZnONPs (50mg/L, 100mg/L, 150mg/L,

200mg/L, and 250mg/L) were applied to hydroponically grown CA, and plant samples were harvested at intervals of 7, 14, 21, and 28 days. The bioactive compounds were detected using reverse-phase high-performance liquid chromatography (HPLC) with an Agilent 1200 system.

This study explores the potential of nanotechnology in enhancing the production of bioactive compounds in medicinal plants, thereby contributing to the field of agricultural nanotechnology. It also addresses the practical challenge of meeting market demand for CA's bioactive compounds, which is critical for the pharmaceutical and cosmetic industries. By optimizing the production of these compounds, the study promotes sustainable agricultural practices and supports the growing trend towards natural and organic products.

2.0 Materials and methods

2.1 Plant material

Stock plants of *Centella asiatica* L. Urban (CA) were purchased from a commercial nursery in Sungai Buloh, Selangor, Malaysia. The nursery is located at approximately 3.1397° N latitude and 101.5923° E longitude and sits at an altitude of around 25 to 50 meters above sea level. The region experiences a tropical climate with high humidity, typically ranging between 80% and 90% year-round. The identification of the species was conducted by the Forest Research Institute Malaysia (FRIM), Selangor, Malaysia, and the plant identification number (PID) assigned is 411218-34.

2.2 Chemicals and reagents

The chemical standards of asiatic acid, madecassic acid, asiaticoside, and madecassoside (purity >=98%) were obtained from Chemfaces (China). The HPLC-grade acetonitrile (LiChrosolv® Reag. Ph Eur) was sourced from Merck (Germany) and phosphoric acid (CAS number: 7664-38-2) was purchased from Nacalai Tesque (Japan).

2.3 Measurement of plant growth

Two weeks before initially starting the experiments, the CA plantlets were vegetatively propagated in the laboratory to gain a uniform and identical size of plants. Once the plantlets had rooted, they were nourished with an essential nutrient solution. At the beginning of the experiments, three homogeneous plants per treatment group consisting of two to three fully expanded leaves were selected and cultivated hydroponically using a basic hydroponic nutrient solution (NS) supplemented with five different concentrations of zinc oxide nanoparticles (ZnONPs), ranging from 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L respectively. The details of the medium were labelled as per in Table 1 below. The plants were grown under 16 hours of photoperiod and 25 degrees of temperature for 28 days. CA plants grown in distilled water and CA grown in a basic hydroponic nutrient solution without any ZnONPs included were used as a control plant.

2.4 Extraction and analysis of triterpenoid derivatives in CA

The leaves and stems of five experimental plants from each treatment group were harvested after 7, 14, 21, and 28 days of treatment application. Immediately after harvest, the plant samples were oven-dried at 40°C for 7 days. Then, the dried samples were ground using a grinder. The samples were soaked with 20 mL of absolute analytical grade

methanol three times for three consecutive days.

Table 1: Composition of different hydroponic growth media for experimental treatments

Medium	Medium Composition
A	Distilled water only
B	Basic hydroponic nutrient solution (NS) only
C	Basic hydroponic nutrient solution (NS) supplemented with 50 mg/L ZnONPs
D	Basic hydroponic nutrient solution (NS) supplemented with 100 mg/L ZnONPs
E	Basic hydroponic nutrient solution (NS) supplemented with 150 mg/L ZnONPs
F	Basic hydroponic nutrient solution (NS) supplemented with 200 mg/L ZnONPs
G	Basic hydroponic nutrient solution (NS) supplemented with 250 mg/L ZnONPs

Then, the samples were filtered, and the filtrate was evaporated to dryness using a rotary evaporator to obtain a crude extract. 10 mg of the residue was dissolved in 1 mL of HPLC-grade methanol, filtered through 0.45 µm membranes, and subjected to HPLC analysis.

2.5 Preparation of standard solutions

Stock methanolic standard solution of four bioactive compounds of CA (asiatic acid, asiaticoside, madecassoside, and madecassic acid) and two internal standards (IS) which were acetonitrile and methanol solution, were separately prepared at a concentration of 1 mg/mL. These stock solutions were kept frozen and used within one week.

2.6 High-Performance Liquid Chromatography (HPLC) analysis

One milligram of the dried crude extract was dissolved in 1.0 mL of absolute HPLC-grade methanol. The extracts were filtered through a 0.45 µm filter prior to running a sample on an HPLC system. The HPLC condition was as follows; analysis was carried out at laboratory temperature with a Column-ZORBAX 300SB-C18 (particle size 5-Micron, size 250 mm x 4.6 mm; from Agilent Technologies), the mobile phase comprised of 0.3% orthophosphoric acid (Solution A) and acetonitrile (Solution B), and the detectors used were a Diode Array Detector (DAD) at UV 206 nm and an Evaporative Light Scattering Detector (ELSD). A 20 µL aliquot of the extract was injected into the column. HPLC analysis was controlled by Chemstation for LC Systems software from Agilent Technologies (2001-2012). The details of gradient elution are shown in Table 2 below.

Table 2: Gradient elution program

Time (Min)	(Solution A) (mL)	(Solution B) (mL)
0.01	95	5
5	80	20
15	50	50
20	20	80
25	20	80
30	50	50
35	80	20
40	95	5

2.7 Statistical Analytical

Data were analyzed using GraphPad Prism version 10 (GraphPad Software, Inc). The values were expressed as the mean ± standard deviation (SD). A one-way ANOVA with Tukey's multiple comparison test was used to establish the statistical significance of the differences. A p-value of less than 0.05 was considered statistically significant.

3.0 Results

The HPLC analysis provided in Figure 1 below illustrates the standard retention times of four key bioactive compounds: madecassoside, asiaticoside, madecassic acid, and asiatic acid, in CA.

The extraction process resulted in crude extract yield of approximately 4%, which was subsequently analyzed for its bioactive compounds (madecassoside, asiaticoside, madecassic acid, and asiatic acid) profile using HPLC. The HPLC chromatogram below provides detailed data on the chemical profile and concentration changes of these four key compounds for CA crude extract treated with various concentrations of zinc oxide (ZnO) nanoparticles over a 7-day period (Figure 2).

Figure 2 shows that after 7 days of ZnONP treatment, madecassoside exhibits a significant peak at 10.43 minutes, with the highest levels in the Medium B and Medium C treatments. Asiaticoside, at 11.3 minutes, and asiatic acid, at 18.5 minutes, also shows a prominent peak, especially in the same treatments. Madecassic acid, at 16.4 minutes, exhibits a moderate peak, highest in the Medium C treatments. The analysis indicates that ZnO nanoparticle treatment significantly enhances the concentrations of madecassoside and asiaticoside, suggesting robust enhancement of their biosynthesis or stability, while the effects on asiatic acid and madecassic acid are positive but less pronounced after 7 days treatment. Notably, no significant peaks were observed for these key compounds in high concentrations of ZnONPs in Medium F and G (200-250 mg/L) treatments, indicating a possible regulatory mechanism that prevents overaccumulation at these higher concentrations.

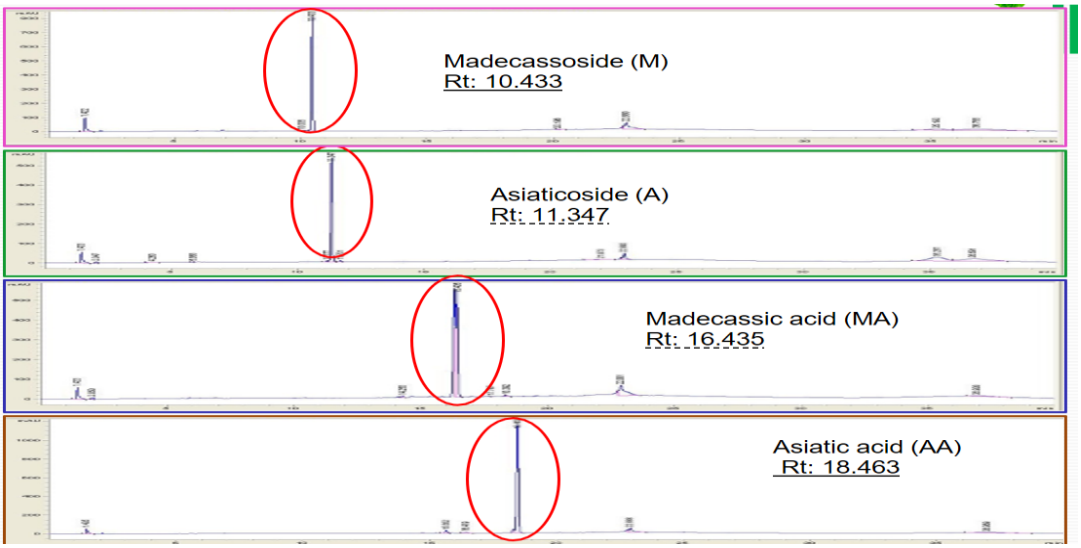

Figure 1: HPLC chromatogram result of standard four bioactive compounds (madecassoside, asiaticoside, madecassic acid and asiatic acid) with their respective retention time (Rt) in CA. The retention times (Rt) for these compounds were as follows: madecassoside at 10.43 minutes, asiaticoside at 11.34 minutes, madecassic acid at 16.44 minutes, and asiatic acid at 18.46 minutes. These Rt were further used to evaluate the production of these four bioactive compounds in hydroponically grown CA treated with five different concentrations of ZnONPs (50mg/L, 100mg/L, 150mg/L, 200mg/L, and 250mg/L).

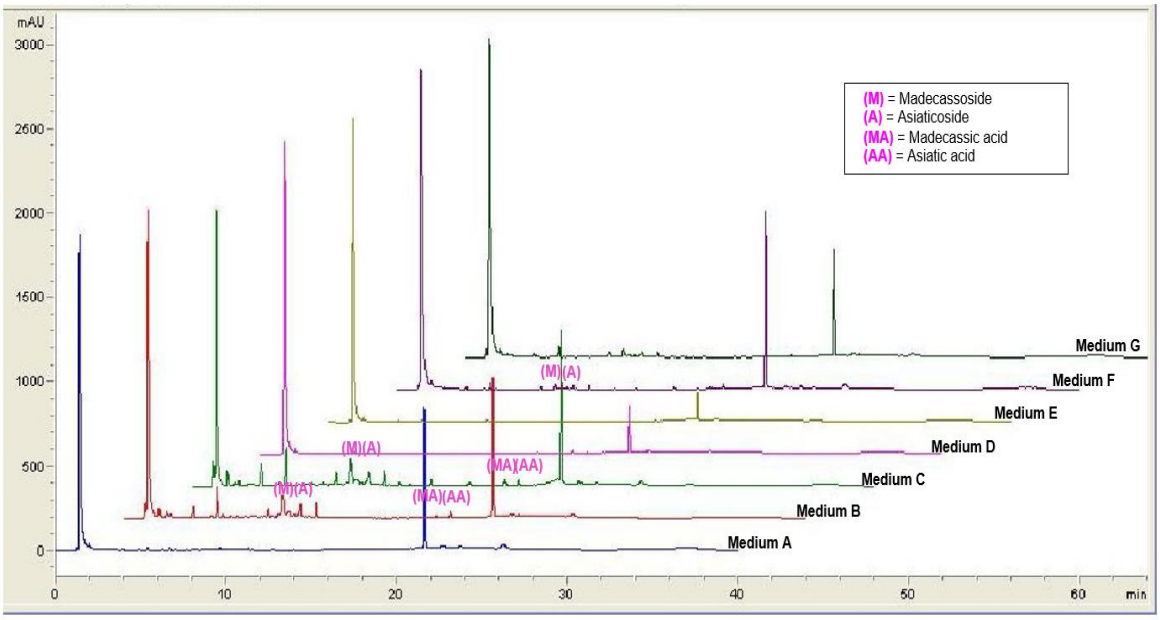

Figure 2: Day 7 HPLC chromatogram of CA crude extract treated with different concentrations of zinc oxide nanoparticles.

After 14 days of treatment with ZnONPs, madecassoside continues to exhibit a significant peak in the chromatogram (Figure 3), similar to the 7-day treatment profile. The consistent peak intensity, or potential increase, suggests that the concentration of madecassoside remains high or even improves with prolonged exposure to ZnONPs. This sustained high concentration implies that ZnONPs effectively enhance the solubility, stability, and biosynthesis of madecassoside over an extended period. The highest peaks for madecassoside were observed in the Medium D and Medium E treatments, indicating these concentrations are optimal for maximizing madecassoside content.

Similarly, asiaticoside also shows a prominent peak, indicating its substantial presence in the treated extract after 14 days. The sustained high intensity or increase in peak size for asiaticoside indicates that ZnONPs stimulating effects on biosynthetic pathways persist over time. The presence of zinc likely continues to act as a cofactor, enhancing the enzymatic activities responsible for producing asiaticoside. The data shows that the Medium D and Medium E treatments exhibited the highest peaks for asiaticoside, suggesting these as the optimal concentrations. On the other hand, asiatic acid, identified at a retention time of 18.5 minutes, presents a noticeable peak at Medium B treatment. This peak, with potential increases in intensity, suggests that ZnONPs positively affect asiatic acid concentration over time. This enhancement can be attributed to the nanoparticles' role in stabilizing asiatic acid or stimulating its biosynthesis. Madecassic acid exhibits a moderate peak, consistent with its profile in the 7-day treatment. The sustained or increased intensity of this peak indicates that ZnONPs continue to enhance the concentration of madecassic acid over the 14-day period. Similar to asiatic acid, the

nanoparticles may stabilize madecassic acid or boost its production, though the impact remains comparatively moderate. The highest peaks for madecassic acid were noted in the Medium B and Medium D treatments. The 14-day HPLC chromatogram shows that treating CA with ZnONPs consistently raises the levels of key compounds in the plant. Madecassoside and asiaticoside show significant increases, while the effects on asiatic acid and madecassic acid are positive but less pronounced. Similar to Day 7, no notable peaks were monitored for these four compounds when exposed to high concentrations of ZnONPs in Medium F and G (200-250 mg/L). Madecassoside shows a moderate peak, indicating a sustained or increased concentration in the treated extracts over 21 days, with the highest peaks in Medium D and Medium E treatments (Figure 4). This suggests that ZnO nanoparticles enhance its solubility, stability, and possibly biosynthesis. Asiaticoside also shows a prominent peak, particularly in the same treatments, indicating ZnO's stimulating effects on its biosynthetic pathways. Meanwhile, both madecassic acid and asiatic acid exhibit a significant peak at Medium B treatments, while only moderate peaks were observed in all ZnONPs treated media, indicating prolong exposure to ZnONPs may have some negative effect on the production of these two compounds.

The HPLC analysis over 28 days shows madecassoside, with a retention time of 10.43 minutes and asiaticoside identified at 11.3 minutes, show noticeable peaks in the medium consist of distilled water only (Medium A), without any basic hydroponic nutrient or any addition of ZnONPs (Figure 5). No significant peaks were observed for asiaticoside and madecassoside in media supplemented with ZnONPs. However, asiatic acid still shows noticeable peaks in the Medium A, and moderate peaks in the Medium D, medium F and Medium G treatments, indicating positive but less pronounced effects on its concentration, possibly through stabilization or enhanced biosynthesis. Only a few small peaks for madecassoside, asiaticoside, asiatic andmadecassic acid were observed in all media containing ZnONPs, specifically medium C, D, E, F, and G (50-250 mg/L ZnONPs).

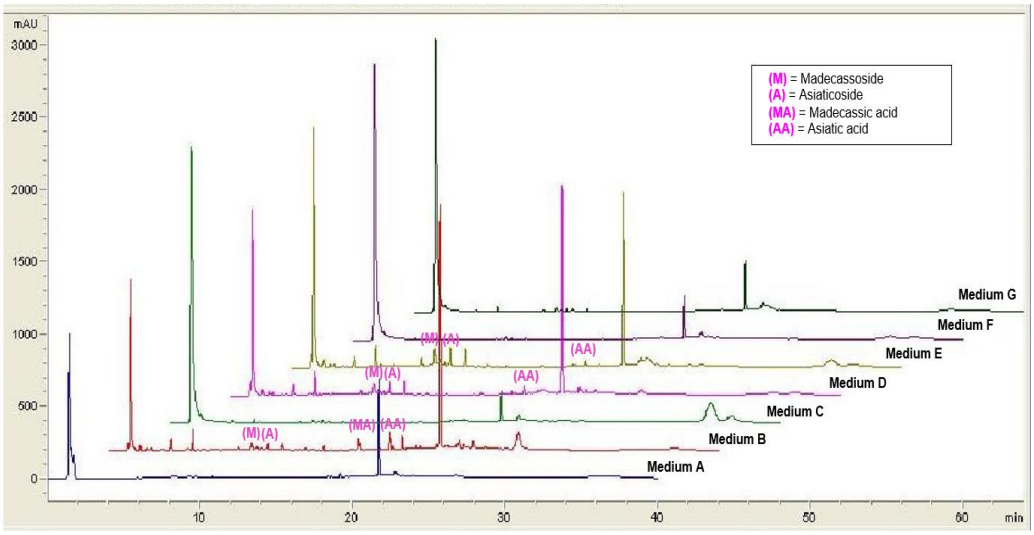

Figure 3: Day 14 HPLC chromatogram of CA crude extract treated with different concentrations of zinc oxide nanoparticles.

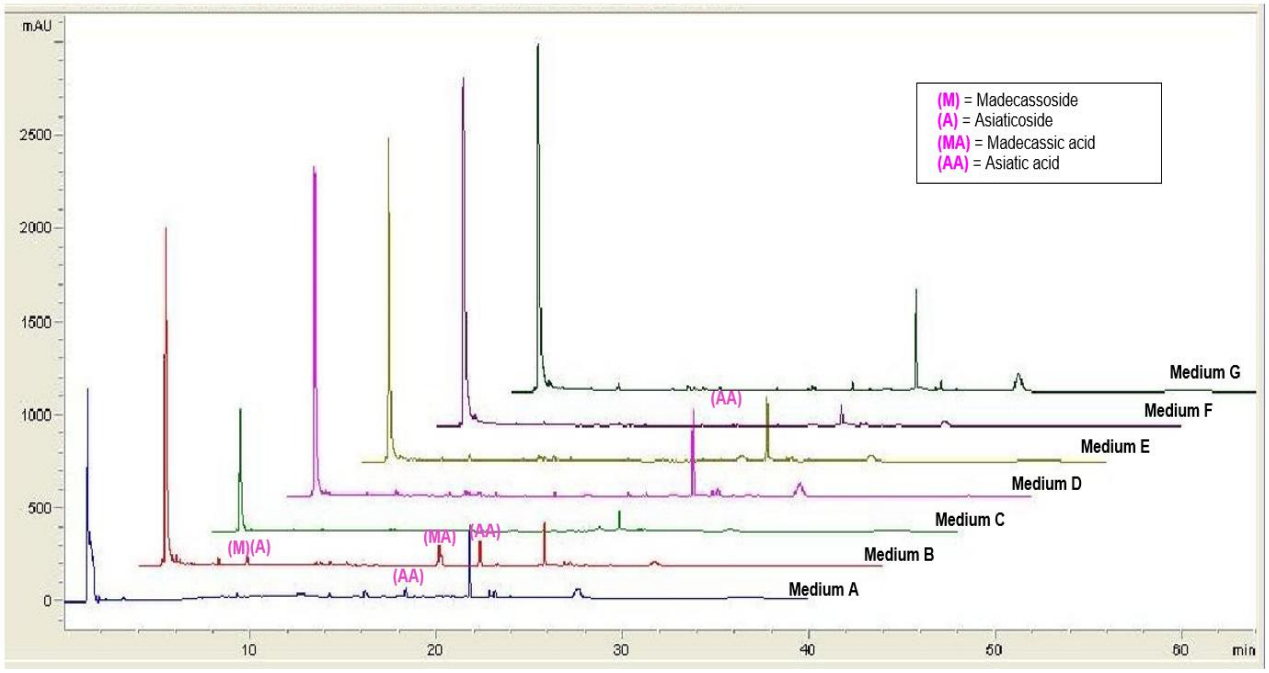

Figure 4: Day 21 HPLC chromatogram of CA crude extract treated with different concentrations of zinc oxide nanoparticles.

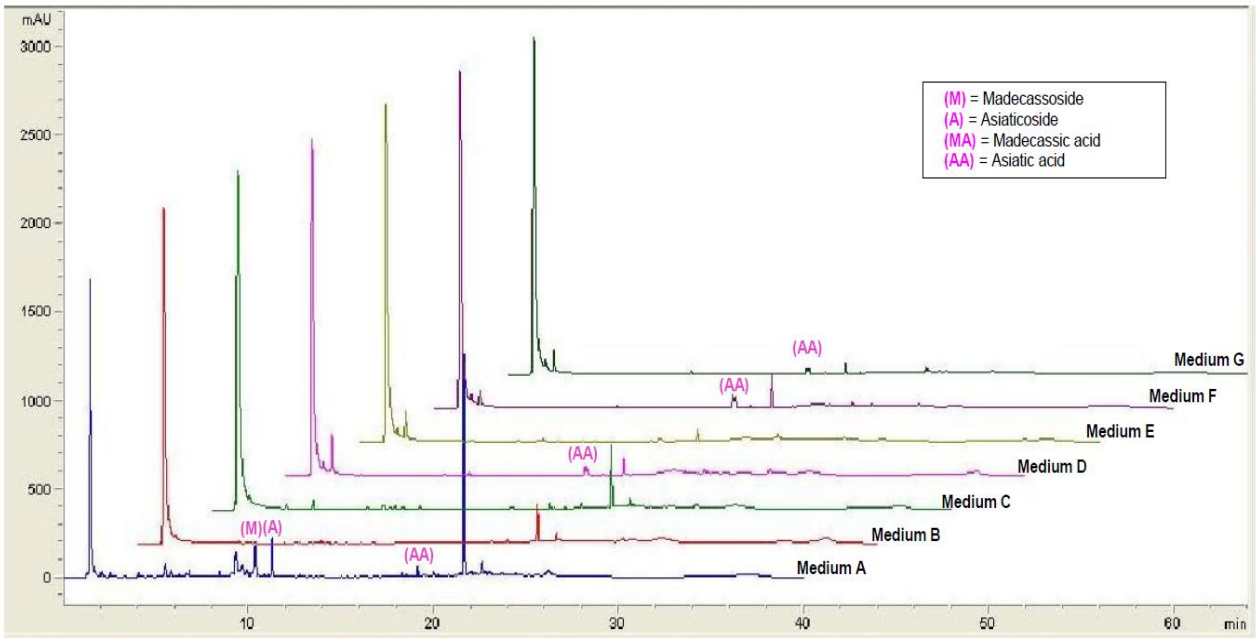

Figure 5: Day 28 HPLC chromatogram of CA crude extract treated with different concentrations of zinc oxide nanoparticles.

4.0 Discussion

The HPLC chromatogram analysis of CA crude extracts treated with ZnONPs over various time periods shows a consistent production trend for key compounds like madecassoside and asiaticoside, especially at ZnO concentrations of 100mg/L to 150mg/L. These compounds exhibited significant peaks in the early stages of treatment (Day 7-14), suggesting enhanced solubility and stability due to ZnONPs (17). However, the production of these compounds did not significantly improve after Day 35, likely due to a saturation effect where initial stimulatory effects plateau (18). Prolonged exposure to high ZnONP concentrations could lead to toxicity, affecting plant metabolism and inhibiting further production (19). Asiaticoside showed similar enhancement patterns, with significant peaks at 100mg/L and 150mg/L ZnO. The production mechanisms are likely similar, involving zinc's role as a cofactor and ROS-induced stimulation (20). Yet, like madecassoside, its production plateaued after Day 35 due to saturation and potential toxicity (21). Asiatic acid, known for its antioxidant and anti-inflammatory properties, also showed peaks in early stages, particularly at higher ZnO concentrations (150mg/L and 200mg/L), but less pronounced compared to the madecassoside and asiaticoside. This differential response may be due to the specific biosynthetic pathways involved. Madecassic acid exhibited moderate peaks at 100mg/L and 150mg/L ZnO, with similar enhancement mechanisms but a less significant response.

The observed enhancement of madecassoside and asiaticoside production in CA at lower ZnONP concentrations (50mg/L to 150mg/L) during the early stages of treatment (Days 7-14) can be attributed to several potential mechanisms. First, nanoparticles can improve the absorption of essential nutrients by plants (22, 23). Zinc, a vital micronutrient, is crucial for various enzymatic functions and metabolic processes in plants (24). ZnONPs might facilitate more efficient uptake of zinc and other minerals, which could enhance the biosynthesis pathways of bioactive compounds like madecassoside and asiaticoside. Enhanced nutrient uptake ensures that the plants have sufficient raw materials required for the production of these secondary metabolites, which are often synthesized as a part of the plant's defensive and adaptive responses (25, 26).

Secondly, ZnONPs may stimulate specific metabolic pathways responsible for the synthesis of these bioactive compounds (27). Nanoparticles are known to induce stress responses in plants, leading to the upregulation of secondary metabolite pathways as a defensive mechanism. This stress response can trigger the plants to produce higher levels of secondary metabolites (28), including madecassoside and asiaticoside, which protect the plant from environmental stressors (29, 30).

Additionally, low concentrations of ZnONPs might enhance photosynthetic efficiency (31) and overall plant growth (32), providing more energy and resources for the production of secondary metabolites. Zinc plays a vital role in the formation of chlorophyll and other pigments, which can improve photosynthesis (33, 34). Improved photosynthetic activity translates to better growth and development, which in turn supports increased production of secondary metabolites (35).

Furthermore, ZnONPs can influence the hormonal balance within plants. They might affect the levels of growth hormones like auxins and cytokinins (36), promoting the

production of secondary metabolites as part of the plant's growth and development process. This hormonal regulation can optimize the plant's physiological state to favor the biosynthesis of beneficial compounds (37).

The results align with previous studies where nanoparticles have been shown to influence secondary metabolite production. For instance, research on *Juniperus procera* callus showed that 5, 20, and 50 mg/L of silver nanoparticles (AgNPs) significantly enhanced biomass accumulation and increased the levels of non-enzymatic antioxidants, including phenols, tannins, and flavonoids. Bioactive compounds such as gallic acid, tannic acid, coumarin, hesperidin, rutin, quercetin, and ferruginol also showed a significant increase in response to AgNPs treatments (38).

A research by Lankarani *et al*. (39) found that iron sulfate and ZnONPs, both individually and combined, increased glycoside content in stevia shoots compared to controls. The highest glycoside levels were achieved with 55.6 mg/L iron and 10 mg/L ZnONPs. ZnONPs at 10 mg/L significantly enhanced iron's effect on glycoside biosynthesis. The maximum stevioside (72.75 mg/L) was produced with 10 mg/L ZnONPs and 27.8 mg/L iron, a 75.04% increase over the control. Rebaudioside also saw the highest increase (63.08%) with this combination. Even higher ZnONPs concentrations (20 and 30 mg/L) with 27.8 mg/L iron-boosted glycoside content, though less effectively than the 10 mg/L ZnONPs combination (39).

The foliar fertilization with ZnONPs and zinc sulfate (ZnSO4) significantly increased capsaicin and dihydrocapsaicin content in Habanero peppers (*Capsicum chinense* Jacq.). The highest capsaicin content (625.44 mg/kg) was at 2000 mg/L ZnONPs, 19.3% higher than the control. Similarly, dihydrocapsaicin increased by 10.9% at the same ZnONP concentration. Total capsaicinoid content, measured by HPLC, was highest (952.15 mg/kg) with 2000 mg/L ZnONPs, a 16.4% increase over the control. ZnONPs also boosted phenol and flavonoid content in *Capsicum chinense* Jacq.) (40). Similarly, foliar application of zinc oxide nanoparticles (ZnO NPs) into plant cultivation improves growth and alters bioactive components in *Curcumin longa*. *C. longa* leaves that were treated with different ZnONPs doses showed increment in the bisdemethoxycurcumin, demethoxycurcumin, and curcumin levels by 2.69-2.84, 2.61-3.22, and 2.90-3.45 times, respectively (41).

The study by Guillen *et al.* (42) showed a significant increase in non-enzymatic antioxidants (vitamin C, anthocyanins, phenols, flavonoids) and antioxidant capacity in grapevine berries in response to zinc application. These increases may result from the plant's defense mechanism against oxidative stress caused by higher zinc levels. Enzyme activities and protein content also changed: 50 mg/L zinc increased catalase activity, and 75 mg/L enhanced peroxidase activity. Zinc acts as a cofactor for enzymes that help plants tolerate oxidative stress, but higher doses can inhibit enzyme activity in grapevine berries (42).

In this research, we noticed that the production of bioactive compounds did not show much improvement after Day 28. This saturation effect suggests that the initial stimulatory effects of ZnONPs has reached a plateau after a certain period (43). The biosynthetic pathways might have been fully activated by the initial doses of ZnONPs, and additional exposure does not lead to further increases in production (44).

These research findings also show that a higher concentration of ZnONPs (200–250

mg/l) lowered the production of madecassoside, asiaticoside, asiatic acid, and madecassic acid as early as Day 7. This indication persisted until Day 28, when all mediums containing ZnONPs showed almost no production of these four key compounds. Many evidences showed that prolonged exposure to high concentrations of ZnONPs could potentially lead to toxicity, negatively affecting the plant's metabolic processes (45, 46). High concentrations of ZnONPs can generate reactive oxygen species (ROS) within plant cells, leading to oxidative stress. This stress can damage cellular components such as lipids, proteins, and DNA, thereby impairing the biosynthetic pathways responsible for secondary metabolite production. An article by Zhang *et al*. (2022) (50) discusses how metal nanoparticles, including ZnONPs, induce oxidative stress in plants, resulting in altered metabolic activities and reduced synthesis of secondary metabolites (44). The plant might respond to this stress by downregulating the production of certain secondary metabolites to prevent toxicity or metabolic imbalances(47).

Plants also have natural regulatory mechanisms that control the production of secondary metabolites. These mechanisms ensure that the accumulation of these compounds does not reach levels that could be detrimental to the plant's normal functions. Once the initial surge in production stabilizes, these regulatory mechanisms might limit further increases, resulting in the observed plateau (48). These mechanisms include feedback inhibition, compartmentalization, hormonal control, gene expression regulation, and environmental feedback (49). Feedback inhibition occurs when the end product of a metabolic pathway inhibits an enzyme involved early in the pathway, preventing overproduction, as highlighted by Zhang *et al*. (2020) (50) in their study on phytosterols (50). Compartmentalization involves the spatial separation of secondary metabolites within specific tissues or cellular organelles, preventing disruption of primary metabolism, as discussed by Dixon and Dickinson (2024) (51). Hormonal control is another key regulatory mechanism, with plant hormones like jasmonic acid, salicylic acid, gibberellins, and abscisic acid playing crucial roles in modulating secondary metabolite production in response to stress, as described by Zong-Yu *et al*. (2021) (52). Additionally, exposure to high concentrations of ZnONPs can lead to changes in the expression of genes associated with stress responses, detoxification, and secondary metabolite biosynthesis. The expression of genes involved in secondary metabolite biosynthesis is tightly controlled at transcriptional and post-transcriptional levels, ensuring optimal production (53). Environmental factors such as light, temperature, and nutrient availability also influence secondary metabolite production, with plants adjusting their metabolic pathways in response to these cues. This was demonstrated in a study by Li *et al*. (2020) (54), which examined the impact of environmental factors on medicinal plants. These regulatory mechanisms collectively ensure that secondary metabolite production is balanced and does not impair normal physiological functions. Moreover, high levels of ZnONPs can inhibit key enzymes involved in the biosynthesis of secondary metabolites, either through direct interaction with the enzymes or by altering the expression of genes encoding these enzymes. Research by Ahmed *et al*. (2022) (55) shows that nanoparticles can affect the activity of specific enzymes crucial for secondary metabolite biosynthesis, thereby reducing the overall production of these compounds.

Overall, the results of this study contribute to the growing body of evidence that nanoparticles can significantly enhance the production of bioactive compounds in medicinal plants. Understanding these mechanisms will provide a solid foundation for developing sustainable and efficient methods for producing high-quality bioactive compounds in various medicinal plants.

5.0 Conclusion

The HPLC analysis of CA crude extracts treated with ZnONPs over 7, 14, 21, and 28 days reveals consistent production trends for key compounds, particularly for madecassoside and asiaticoside, with optimal enhancement observed at ZnO concentrations of 100mg/L to 150mg/L. Although positive, the effects on asiatic acid and madecassic acid are less detected, indicating a differential response to ZnONP treatment. Higher concentrations of ZnONPs (200–250 mg/L) led to a considerable decrease in the formation of madecassoside, asiaticoside, asiatic acid, and madecassic acid starting from Day 7. The trend persisted until Day 28, when all medium containing ZnONPs exhibited minimal synthesis of these four bioactive compounds. This study demonstrates that ZnONPs can significantly enhance the production of asiaticoside and madecassoside in CA, with optimal concentrations ranging from 50mg/L to 150mg/L from as early as 7 days upon treatment, showing the potential of nanotechnology to improve the yield of valuable secondary metabolites in medicinal plants. The findings suggest that ZnONPs, like other nanoparticles, can improve nutrient uptake, stimulate metabolic pathways, enhance photosynthesis, and regulate hormonal balance, leading to increased production of valuable secondary metabolites in CA. These insights can be applied to optimize the cultivation of CA and other medicinal plants to meet the growing demand for natural bioactive compounds in the pharmaceutical and cosmetic industries. Future research should continue to explore the mechanisms behind nanoparticle-induced enhancements and evaluate the long-term effects of nanoparticle treatment on plant health and compound stability.

Authorship contribution statement

NAS: Data analysis, Methodology, Formal analysis, Writing–original draft. **HFM**: Visualization, Methodology, Writing – review & editing. **MR**: Visualization, Resources, Draft corrections. **MSAN**: Supervision, Funding acquisition, Writing – review & editing.

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Conflict of Interest

The authors declared that they have no conflicts of interest to disclose.

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