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Research Article

Antihypertensive activity of spray-dried nanoemulsion containing Asiatic acid-Palm oil in high salt diet-fed rats

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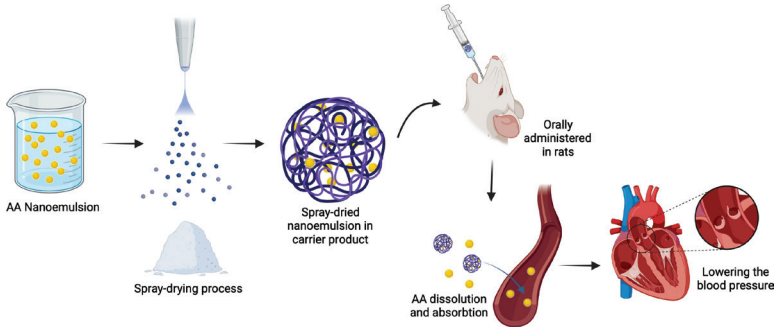
Abstract

Asiatic acid (AA) is a compound isolated from *Centella asiatica*, which possesses significant antihypertensive activity. Several studies have shown that its hypertensive activity can be attributed to various mechanisms, such as Angiotensin-Converting-Enzyme (ACE) inhibition in the renin-angiotensin-aldosterone system (RAAS) pathway. Meanwhile, palm oil (PO) is an antioxidant, which has proven to have synergistic effects with the compound by preventing arterial thrombosis and atherosclerosis. Despite these synergistic effects, AA dosage in antihypertensive therapy has been reported to be relatively high compared to the common synthetic drug captopril. Therefore, this study aimed to produce spray-dried powder of nanoemulsion to enhance the solubility of AA, decrease the possibility of oxidation, and increase its activity. Redispersed AA nanoparticles were also successfully obtained during the synthesis process. Several evaluations were carried out, including particle size, particle distribution, zeta potential, cell viability, and antihypertensive activity in rats to ensure the improvement of physicochemical characteristics and activity as antihypertensive agent. The results showed that AA succeeded in forming nanoemulsion with excipients. In addition, it was encapsulated in a maltodextrin carrier, exhibiting good physicochemical characteristics and safety to the Caco-2 cells. The redispersion of the spray-dried powder yielded nanoparticles with a size of 217.4 ± 10.196 nm. The spray-dried nanoemulsion of AA also had faster effect than non-formulated AA (raw powder) in lowering the blood pressure of hypertensive Sprague-Dawley (SD) rats.

Graphical abstract:



Keywords

Asiatic acid, Palm oil, Antihypertensive, Spray-dried nanoemulsion, ACE-inhibitor

Introduction

Hypertension is a non-communicable disease (NCD) that has long been known as a global problem. This disease is typically caused by high blood pressure in the arteries and poses substantial risks, including heart disease, stroke, cardiovascular complications, and renal diseases (Mills et al. 2016; Giena et al. 2018; Sakboonyarat et al. 2019). According to previous studies, addressing hypertension necessitates a comprehensive understanding of various mechanisms, with a crucial focus on regulating the renin-angiotensin-aldosterone system (RAAS). In addition, primary therapeutic interventions commonly comprise the use of angiotensin-converting enzyme inhibitors (ACEi), angiotensin II receptor blockers (ARBs), calcium channel blockers (CCB), thiazide diuretics, and β-adrenoceptor antagonists (β - blockers) to effectively manage blood pressure (Neal 2016; Saseen and MacLaughlin 2017). Several natural medicines, such as Asiatic acid (AA) and palm oil (PO) have also been developed as antihypertensive agents to treat this disease (Ganafa et al. 2002; Bayorh et al. 2005; Bunbupha et al. 2014; Maneesai et al. 2016, 2017; Meeran et al. 2018; Fong et al. 2019).

Among these agents, AA, isolated from *Centella asiatica* commonly found in tropical countries (Committee on Herbal Medicinal Products (HMPC) 2010; Fong et al. 2019), exhibits antihypertensive pharmacological activity through various mechanisms (Bunbupha et al. 2014; Pakdeechote et al. 2014; Maneesai et al. 2016, 2017; Meeran et al. 2018; Fong et al. 2019). This compound has been reported to have the ability to reduce blood pressure by enhancing nitric oxide pathways, attenuating RAAS pathways by angiotensin II and ACE activity inhibition in blood, decreasing reactive oxygen species (ROS) formation, and forming cytoprotective activity (Bunbupha et al. 2014; Maneesai et al. 2016, 2017; Meeran et al. 2018; Fong et al. 2019). However, AA has several limitations, including low solubility in water (around 0.1583 mg/mL in saturated saline), absorption by passive diffusion and metabolism by the CYP450 enzyme, leading to decreased bioavailability in the blood (Yuan et al. 2015; Meeran et al. 2018; Islamie et al. 2023).

Another essential antihypertensive agent is PO, a vegetable oil derived from the *Elaeis guineensis* plant. Several studies have shown that PO is rich in vitamins A and E and has no lipid-raising fatty acids in its saturated fatty acid content (Ganafa et al. 2002; Bayorh et al. 2005). This oil also has antihypertensive properties with AA due to its strong antioxidant activity, showing efficacy in reducing arterial thrombosis, atherosclerosis, and platelet aggregation (Ganafa et al. 2002). However, the oxidation process can reduce the vitamin content of PO, prompting the need for a nanoencapsulation system to ensure content stability (Ricaurte et al. 2017; Sandoval-Cuellar et al. 2020). The formation of a nanoparticle system can be used to overcome the limitations of AA related to physicochemical characteristics and pharmacokinetic aspects (Sheng 2009; Nirmala and Nagarajan 2017; Mehmood et al. 2019).

According to previous reports, the ACE inhibition activity of AA is lower compared to captopril, a well-known ACE inhibitor compound, even when administered at a 6-fold higher dose (Maneesai et al. 2017). Therefore, this study aimed to synthesize nanoemulsion-loaded AA-PO to increase antihypertensive activity through ACE inhibition. The spray drying process of the nanoemulsion in maltodextrin was carried out to obtain the benefits of encapsulation and reduce the possibility of oxidation (Freers 2009; El-Messery et al. 2020). Several evaluations, such as particle size, zeta potential, cell viability, and non-invasive antihypertensive tests were performed to measure the improvement of AA characteristics and its activity. The spray-dried nanoemulsion effectively reduced the particle size of AA when redispersed in water, leading to increased solubility and dissolution rate (Pradana and Ritthidej 2023). The improvement in pharmacokinetic profile and bioavailability (drug amount in blood) led to an increase in the efficacy of antihypertensive therapy (Shargel and Yu 2016).

Materials and methods

Materials

Asiatic acid (AA) (≥ 95%) used in the formulation was supplied by New Natural Biotechnology Co., Ltd, (Shanghai, China) and the palm oil (PO) was from Sigma-Aldrich (St. Louis, Missouri). Excipients with pharmaceutical grade used in the study were Tween 80 provided by Maximax Pro Co., Ltd (Bangkok, Thailand), soy lecithin from Sigma-Aldrich (St. Louis, Missouri), maltodextrin by Sigma-Aldrich (St. Louis, Missouri), and magnesium stearate supplied by S. Tong Chemicals (Nonthaburi, Thailand). Other materials included captopril from TCI (Shanghai, China), absolute ethanol supplied by Emsure, Merck Millipore, Co., (Darmstadt, Germany), Fetal Bovine Serum (FBS) Gibco from Life Technologies Ltd (Paisley, UK), Penicillin (10,000 units/mL)-Streptomycin (10,000 µg/mL) Gibco purchased from Life Technologies Ltd (Paisley, UK), L-glutamine 200 mM (100X) Gibco from Life Technologies Ltd (Paisley, UK), Dulbecco's Modified Eagle Medium (DMEM) powder Gibco supplied by Life Technologies Ltd (Paisley, UK), Phosphate-Buffered Saline (PBS) Tablets Gibco by Life Technologies Ltd (Paisley, UK), 0.25%Trypsin-EDTA (1×) Gibco purchased from Life Technologies Co. (NY, USA), sterile dimethyl sulfoxide (DMSO) pro-

vided by Sigma-Aldrich (St. Louis, Missouri), MTT dye Invitrogen from Life Technologies Limited (Paisley, UK), and zetasizer capillary cells (Malvern DTS 1070, UK).

Methods

Molecular docking investigation

This investigation was carried out to predict possible interaction between Asiatic acid (AA) or captopril, with targeted receptors. The 3D structures of compounds (AA and captopril) and target protein (ACE PDB ID 1O86) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/), and RCSB PDB (http://www.rcsb.org/), respectively. Water molecules and lisinopril were removed from the protein 3D structure obtained using Pymol 2.5, and the molecular docking between AA and captopril with ACE was performed by using Pyrx 0.8. Furthermore, the conformation with the highest negative binding energy was selected and the docked complex was converted to a 2D structure to examine the interactions formed at the binding site of 1O86 (targeted protein) with AA and captopril by using BIOVIA Discovery Studio Visualizer 2021 (Biovia, San Diego, CA, USA) (Seeliger and de Groot 2010; Dallakyan and Olson 2015).

Preparation of spray-dried nanoemulsion

The formulation started with the production of a stable nanoemulsion, followed by a spray dry process, and a dry form was obtained. Nanoemulsion was constructed by mixing aqueous and oil phases, and 0.3 g of Tween 80 was initially dissolved in 24.7 mL of deionized water. In the oil phase, 1.0 g of palm oil (PO) was melted at 70 °C and added with 1.0 g of lecithin and AA (0.2 g in 9.8 mL ethanol) solution. Furthermore, the homogenization process (ultraturrax, IKA T25 digital) started at 5,000 rpm, and the aqueous phase was added dropwise into the oil phase. The speed of the instrument was later increased to 10,000 rpm for 5 mins. The homogenization process was continued with a probe sonicator (Sonics Vibra-cell) at 13 W and 60% amplitude for 7 mins to obtain nanoemulsion. A total of 2.945 g of maltodextrin as a carrier and 0.055 g of magnesium stearate as a lubricant were added with stirring at 5,000 rpm. The liquid product was dried using a spray dryer (Buchi Spray Dryer B-290) at 70 °C inlet temperature, 40 mm (473 L/hr) airflow, 90% (35 m^3/hr) aspirator rate, and 5.5 mL/min liquid flow.

Particle size, distribution, and zeta potential

The particle size and distribution measurement of raw AA powder and spray-dried powder of AA nanoemulsion were performed using morphologically directed Raman spectroscopy (MDRS) (Malvern Morphologi 4-ID) using the dry method. The pressure used in this process was 3 bars and the analysis was carried out on calibrated magnification. Therefore, to obtain particle size distribution, the span value was calculated from D90, D50, and D10 data, followed by computation as (D90-D10)/D50.

The particle size (z-average diameter), polydispersity index, and zeta potential of AA nanoemulsion and redispersion of spray-dried powder in water were evaluated using Zetasizer (Malvern Instruments Nano ZS). Furthermore, the test was carried out in triplicate with a sample dilution of 1:100 in deionized water to avoid multiple scattering effects during analysis (Carpenter and Saharan 2017).

Cell viability

Caco-2 cells used in this evaluation were routinely sub-cultured and seeded. The old media was removed and washed with PBS (1×, pH 7.4) and 1,000 µL trypsin (0.25%Trypsin-EDTA 1×). Subsequently, fresh complete DMEM media, heated FBS, penicillin (10,000 units/mL)-streptomycin (10,000 µg/mL), and L-glutamine 100 mM (100X) were added and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Caco-2 cells with passage numbers 20 to 30, in optimum cell growth, were seeded and used in the cytotoxic test.

Cell growth was assessed microscopically for MTT assay and 10 µL was seeded in a 96-well plate (8000–10,000 cells per well). Each well was filled with 10 µL of placebo or samples of different concentrations in a complete medium (0, 0.5, 5, 50, 100, 250, and 500 µM). For samples of high AA concentrations (250 and 500 µM), a small amount of DMSO (< 1% v/v) was added in a complete medium to facilitate the dissolution process. After 24 hours of incubation, the media was replaced with MTT solution (0.5 mg/mL) and incubated with light protection for another 3 hours at 37 °C before replacement of MTT solution by DMSO. The samples were then analyzed using a microplate reader (CLARIOstar, BMG LABTECH) at 570 nm, which showed the absorbance of each well. Cell viability due to the influence of AA, AA nanoparticles, and matrix was obtained as a percentage of control.

Animal study

The protocols in this study were conducted in line with the standards for the care and use of experimental animals and approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (No. 21-33-009). A total of 40 adult Sprague-Dawley (SD) male rats (9 weeks old) supplied by Nomura Siam International, (Bangkok, Thailand), were housed in a 12-hour dark/light heating, ventilation, and air conditioning (HVAC) system. After a week of habituation, the animals were administered a high salt (2% NaCl) diet to induce hypertension for 10 weeks (Cruz et al. 2011). Treatment was started after the blood pressure and ACE1 activity of the HSD-fed rats increased. This starting point was shown in Fig. 1A at day 70 and was known as the hypertension level limit.

The animals were randomly separated into 1 non-hypertensive group and 4 treatment groups (n = 8 per group), and administered AA, AA nanoparticles, captopril, or matrix (nanoparticles with no AA) daily. For 3 consecutive weeks, AA, AA nanoparticle (equal to 30 mg/kg/day of AA), matrix, or captopril (5 mg/kg/day) was administered orally, as shown in Fig. 1B (Maneesai et al. 2017). Captopril was selected as a positive control because it was an ACE inhibitor that was recommended for first-line therapies for hypertension (Kovell et al. 2015). The systolic blood pressure was measured weekly using a non-invasive tail-cuff plethysmography (AD Instrument Powerlab 8/35 and NIBP controller) (Fig. 1C) to determine blood pressure fluctuations.

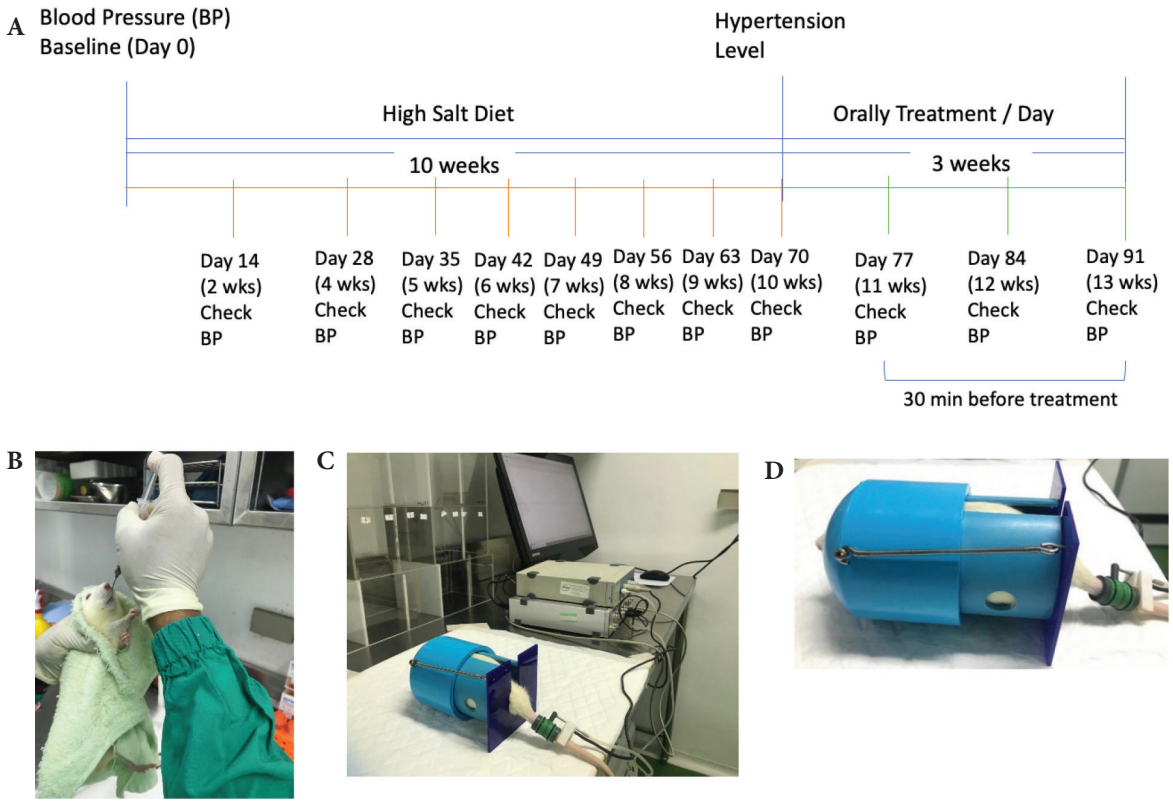


Figure 1. Antihypertensive activity study in rats: Study timeline (**A**), Oral administration (**B**), Non-invasive blood pressure instrument (**C**), Animal handling on indirect blood pressure test (**D**).

On each evaluation day, the animals were restrained and allowed to acclimatize before their blood pressure was measured, as shown in Fig. 1D. The instrument amplifier provided pulse amplitude and the blood pressure obtained was recorded. The measurement was conducted 3 times and the average values for each rat were calculated.

ACE1 activity

Blood was obtained from the tail vein of the rats at week 9 of hypertension induction and directly from the heart at the end of the treatment process, as shown in Fig. 2. The collected blood of 0.5 ml was centrifuged to obtain plasma. Subsequently, the ACE1 assay buffer, substrate, and enzyme were prepared. Serum from all rats of 0.03 mL was used as a sample on a 96-well clear bottom UV plate, while ACE1 assay buffer was used as a blank, with ACE1 enzyme serving as a positive control. Each solution in the well was adjusted to 200 µL with ACE1 assay buffer, incubated for 10 mins at 37 °C, and then added with 50 µL of ACE1 substrate (diluted 5 folds with buffer). The bubbles formed after mixing were removed, and the absorbance was read with a microplate reader (CLARIOstar, BMG LABTECH)

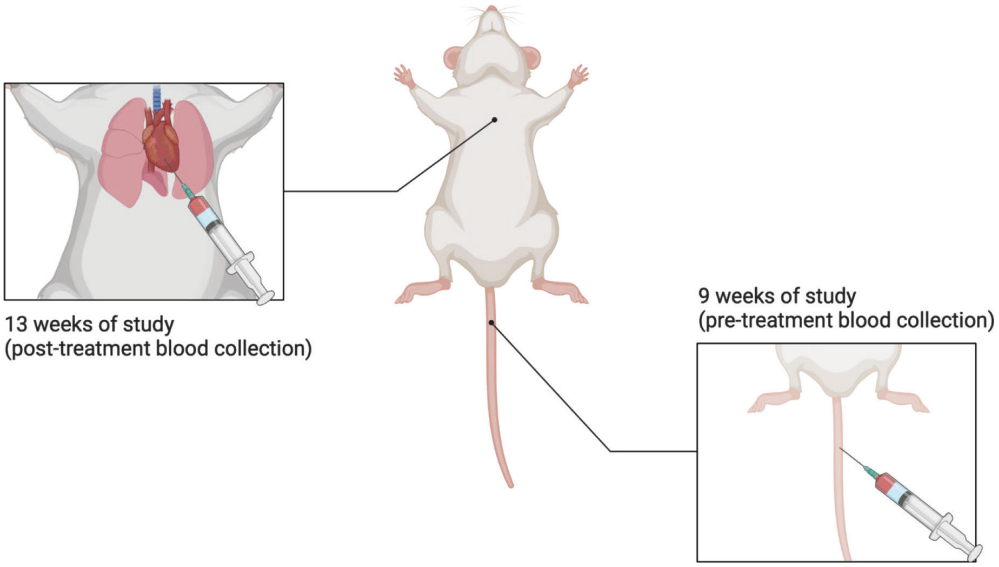


Figure 2. Rat blood collection site at pre-and post-treatment in animal study.

at 345 nm for 60 mins. Serum absorbance and blank were used to calculate ACE1 activity in all groups.

Statistical analysis

Data of triplicate evaluation were shown as mean ± SD. For the in vivo study, results were obtained from 6–8 rats/groups. The differences among groups were tested using an unpaired t-test for particle size, z-average, polydispersity index, and zeta potential. Meanwhile, 2-way ANOVA was used for blood pressure results, and ordinary 1-way ANOVA was used for ACE1 activity results. Furthermore, the analysis was followed by Tukey's test and considered to be statistically significant when the p-value was < 0.05. All statistical analyses were carried out with GraphPad Prism Version 9.4.0.

Results and discussion

Molecular docking investigation

To investigate whether ACE activity could be due to direct interaction between AA and standard ACE inhibitor (captopril), a molecular docking study showed that both compounds were bound to the ACE, as shown in Fig. 3. The key interactions to stabilize the complex were hydrogen bonding, Van der Waals, and hydrophobic interactions, as listed in Table 1. The results showed that AA and captopril consisted of 4 hydrogen interactions and 2 hydrophobic interactions with ACE. Based on these interactions, the free binding energy between AA and captopril with ACE was – 9.7 kcal/mol and -5.6 kcal/mol, respectively (Table 2). The data indicated that AA had a potential interaction with ACE and could interfere with its activity.

Particle size, distribution, and zeta potential

The spray drying of AA nanoemulsion successfully formed a solid product with a size of 4.50 ± 0.27 µm in the dry method determination. This solid product was formed by previous studies, with a yield of more than 70% and a moisture content in the range of 4–6% (Pradana and Ritthidej 2023). The particle size was smaller than raw AA powder, which was 6.05 ± 0.430 µm in size, as shown in Fig. 4A. Based

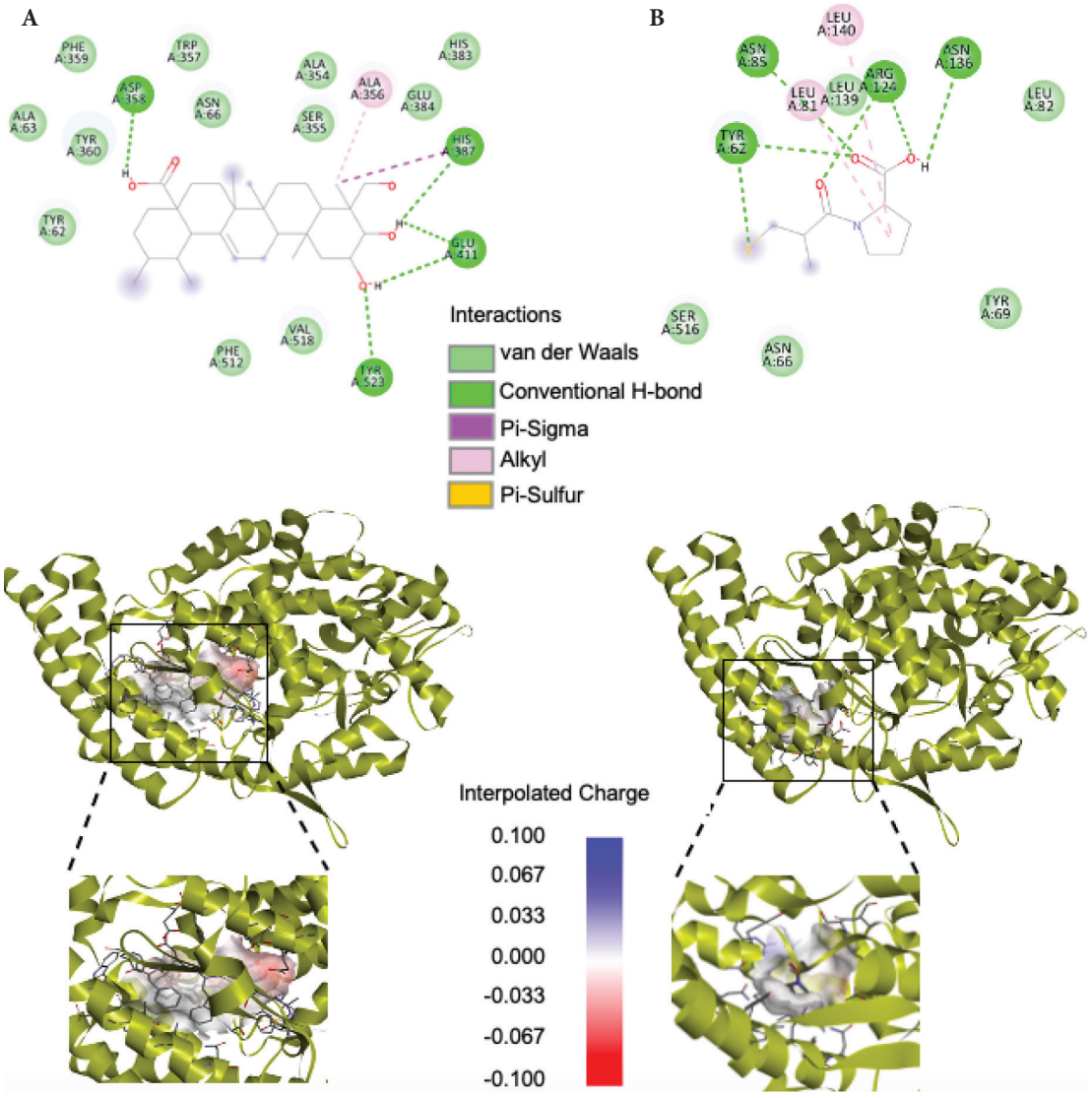


Figure 3. 2D and 3D interaction between Asiatic acid (**A**) and Captopril (**B**) with ACE (PDB ID: 1O86).

Table 1. Type of interaction between Asiatic acid and captopril with ACE (PDB ID: 1O86).

Compound	Hydrogen	Van der Waals	Hydrophobic
Asiatic acid	ASP358	TYR62	ALA356
	HIS387	ALA63	HIS387
	GLU411	ASN66	
	TYR523	ALA354	
		SER355	
		TRP357	
		PHE359	
		TYR360	
		HIS383	
		GLU384	
		PHE512	
		VAL518	
Captopril	TYR62	ASN66	LEU81
	ASN85	TYR69	LEU140
	ARG124	LEU82	
	ASN136	LEU139	
		SER516	

Table 2. Binding energy between Asiatic acid and captopril with ACE (PDB ID: 1O86).

PDB	Binding energy (kcal/mol)	
1O86	Asiatic acid	Captopril
	-9.7	-5.6

on the span value, the uniformity of particle size after being processed was relatively superior, with a value closer to 1.00 (1.27 ± 0.085). This result showed that the spray drying process was able to encapsulate AA nanoemulsion droplets in a hydrophilic carrier without increasing the particle size of the final product, showing the improvement of interfacial contact in the dissolution media.

Fig. 4B–D showed the particle size, polydispersity index, and zeta potential of nanoemulsion and redispersed AA spray-dried product obtained by the wet method. The particle size of the redispersion in deionized water (216.40 ± 2.67 nm) was equivalent to pre-spray-dried nanoemulsion (217.40 ± 10.20 nm), indicating that the drying process with maltodextrin did not inhibit the ability of aqueous solvent to redisperse the dried sample to a nanometer scale (Mahdi et al. 2020). These results were similar to those of the previous report, stating that TPGS-stabilized nanoparticles experienced solubility and bioavailability improvement with approximately a 7-fold increase in AUC after particles of > 7 μm were reduced to 210 ± 0.08 nm in size upon dispersion in water (Rachmawati et al. 2017).

Encapsulation of nanoemulsion in maltodextrin, which is a nonionic polymer, was also shown from the particle distribution and zeta potential value changes (Mahdi et al. 2020; Ribeiro et al. 2020). The zeta potential of pre and post-spray-dried products was more negative than -30 mV, showing high resistance toward particle aggregation. The negative charge at the zeta potential value was due to the fatty acid of the oily phase used in the formulation and stabilized the redispersed AA nanoemulsion by electrostatic repulsion (Páez-Hernández et al. 2019; Jesser et al. 2020). Furthermore, the spray drying process with malto-

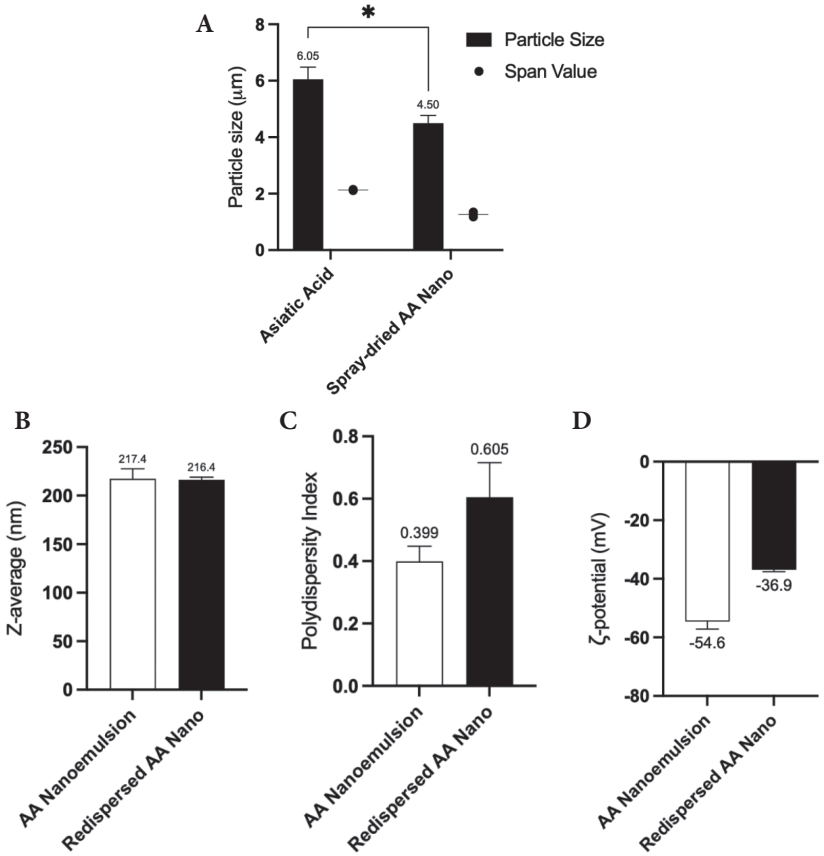


Figure 4. Particle size and distribution with dry method determination (**A**); *showed statistical difference with $p < 0.05$; and wet method determination of particle size (**B**), particle distribution (**C**), and z-potential (**D**).

dextrin successfully reduced the zeta potential value from -54.6 mV to -36.9 mV, showing cell toxicity reduction (Wan et al. 2020; Weiss et al. 2021).

Cell viability

The results of the MTT assay showed Caco-2 cells viability after contact with matrix, AA, and AA nanoparticles, as shown in Fig. 5. The 3 groups (matrix, AA, and AA nanoparticles) remained non-toxic in concentrations up to 100 µM. At higher concentrations, the 3 groups showed different toxicity levels (Fig. 5A) and IC_{50} values (Fig. 5B). The level of the sample toxicity in Caco-2 cells was ranked as AA > AA nanoparticles > matrix. Based on the toxicity values and IC_{50} of AA nanoparticles, which were almost equal to AA, nanoemulsion preparation and the spray drying process did not affect the toxicity to Caco-2 cells (Róka et al. 2015). These results presumed that AA nanoparticles administered orally with an equal dose of AA as antihypertensive agent did not cause toxicity increment in cells.

Antihypertensive activity in animal

Antihypertensive activity was examined in 5 groups of SD rats. This study started with the induction of 2% NaCl in drinking water, which led to a significant increase in blood pressure after 4 weeks. The high salt diet (HSD) was continued until week 9 when constant blood pressure was achieved and confirmed by data on ACE activity improvement, compared to the non-hypertensive group. At this time, oral administration of matrix, AA, and AA nanoparticles were started.

Fig. 6 showed changes in the body weight of rats over 13 weeks. The results showed that the body weight of the non-hypertensive group increased significantly compared to other groups since the first week of high-salt diet induction. A previous study obtained a similar profile of changes in body weight. Lower body weight in high-salt groups was influenced by changes in energy balance for metabolism, where energy expenditure increased due to a long-term salt diet (Coelho et al. 2006).

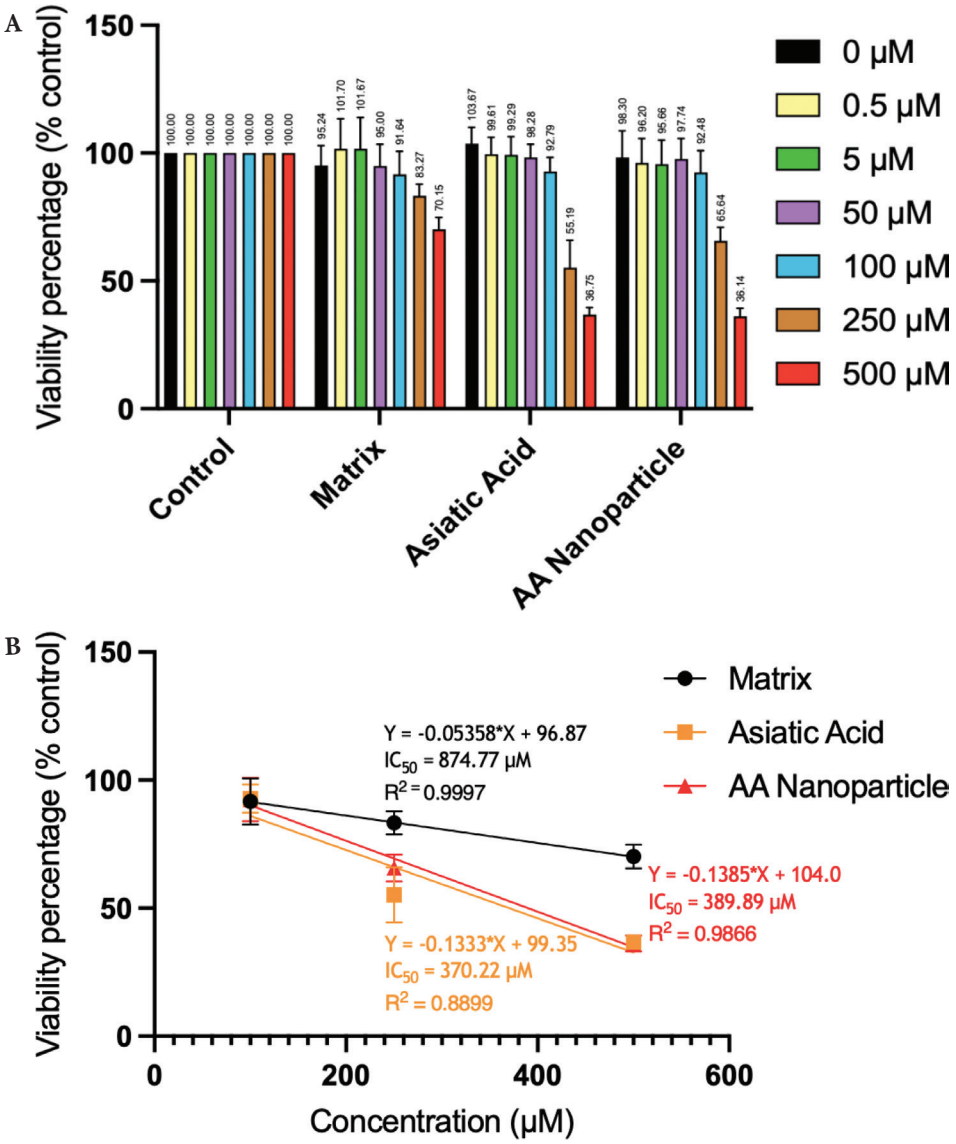


Figure 5. Viability of Caco-2 cells (**A**) and the IC_{50} value (**B**) with matrix, Asiatic acid, and Asiatic acid nanoparticles.

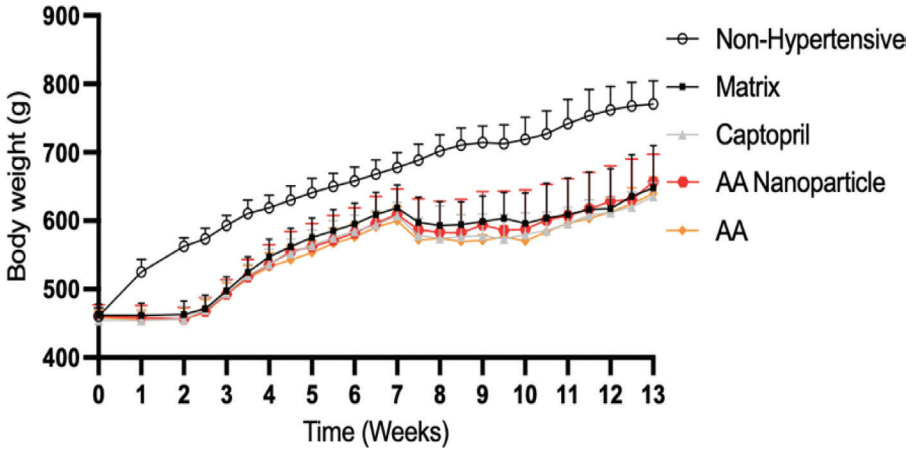


Figure 6. Body weights of 5 experimental groups for 13 weeks.

Systolic blood pressure value changes of all groups are presented in Fig. 7A. From week 4 to week 10 of the HSD program, all groups experienced a significant increase in blood pressure compared to the non-hypertensive group before treatment. Immediately after the treatment started, the rats in captopril and AA nanoparticles groups showed blood pressure reduction with a significant value to the matrix-only group. Blood pressure reduction in the AA group was only seen as significant in the 12th week until the end of the experiment. These results were consistent with a previous study, where AA could reduce systolic blood pressure, but not as fast as captopril (Maneesai et al. 2017). Meanwhile, AA nanoparticles caused a reduction among the hypertensive rats faster than the AA group, at a rate that was almost similar to the captopril group.

ACE-activity of blood serum was assessed to confirm systolic blood pressure reduction. Fig. 7B showed that the ACE activity of all groups at the end of experimental time was still higher ($p < 0.05$) compared to the non-hypertensive group (0.043 ± 0.005 U/ml). In the AA and AA nanoparticles groups, there was no significant decrease compared to the matrix group, with values of 0.057 ± 0.004 and 0.055 ± 0.005 U/ml for the AA group and AA nanoparticle groups, respectively. Meanwhile, captopril decreased ACE-activity value to 0.053 ± 0.004 U/ml, which was significant ($p < 0.05$) against the matrix group of 0.062 ± 0.007 U/ml.

Animal studies showed that AA had ACE inhibitory activity similar to captopril, which could influence the RAAS pathway. The insignificant result on ACE inhibition showed that other mechanisms of lowering blood pressure, such as enhanced nitric oxide pathway or lowered reactive oxygen species (ROS) formation could be considered in further evaluation of post-formulation of nanoparticles (Maneesai et al. 2016; Sulistyowati et al. 2019). In addition, the spray-dried nanoemulsion formulation of AA succeeded in increasing blood pressure reduction and ACE inhibition activity in HSD-fed rats compared to the AA powder form (Gu et al. 2008; Maneesai et al. 2017).

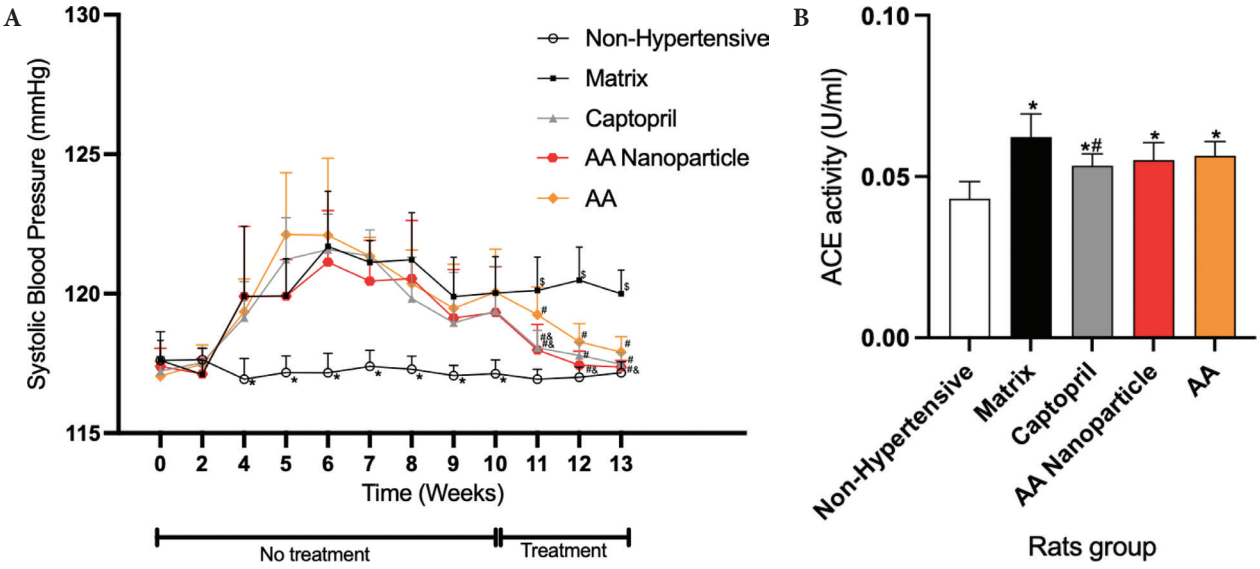


Figure 7. In-vivo antihypertensive activity of SD rats (n = 6-8/group); Systolic blood pressure result of 10 weeks induction and 3 weeks treatment period (**A**), and ACE1 activity post-treatment in serum result (**B**); Showed statistically different ($p < 0.05$) for *between non-hypertensive group with all groups, $^{\#}$between treatment group with matrix groups, $^{\$}$between matrix group with non-hypertensive groups, $^{\&}$between captopril and AA nanoparticles groups with AA group.

Conclusion

In conclusion, the AA nanoemulsion was successfully formed on a nanoparticle scale. The AA nanoemulsion was physically encapsulated in maltodextrin as a spray-dry carrier and immediately formed nanoparticles after being redispersed in an aqueous solvent with a size of 216.40 ± 2.67 nm. Furthermore, the preparation process and the matrix used were also safe and non-toxic to cells, as observed using Caco-2 cells. The molecular docking results showed that AA had ACE inhibition activity. 2% of NaCl in drinking water successfully induced the treatment group by increasing the systolic blood pressure and ACE-activity values. AA nanoparticles succeeded in significantly lowering systolic blood pressure compared to AA raw powder. AA nanoparticles also showed ACE-inhibition of serum, although this mechanism as an antihypertensive agent had not shown a significant result compared to AA raw powder. Based on these findings, the microparticle formulation with high speed of stirring, sonication, and spray drying was successful. This system was redispersed quickly in an aqueous solvent, the AA particle size was reduced to a nanometer, and its activity was optimized as antihypertensive agent.

Disclosure statement

There was no potential conflict of interest reported by the authors.

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