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Aqueous Extract of *Ampelopsis Cantoniensis* With the Addition of *Curcuma Longa* and *Taraxacum Officinale*: LD_{50} on *Zebrafish* (*Danio Rerio*) Embryos and Nanoformulations for Bacteria Inhibition

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ABSTRACT

Herbal extracts are promising sources of bioactive compounds with potential antimicrobial and biomedical applications. However, their safety and bioavailability often limit their practical use. This study aimed to evaluate the acute toxicity and antibacterial activity of an aqueous extract of *Ampelopsis cantoniensis* combined with *Curcuma longa* and *Taraxacum officinale* (Am-CT extract), and to enhance its performance through nanoformulation. The Am-CT extract, containing 29.69% dihydromyricetin, was tested for toxicity in *zebrafish embryos* at concentrations ranging from 1.875 to 15 g L^{-1}. The results indicated that the extract was safe, with an LC_{50} value of 7.81 g L^{-1}, showed weak teratogenicity (EC_{50} = 7.422 g L^{-1}), and had a therapeutic index (TI) of 1.025. Three Am-CT nanoformulations were prepared using chitosan (CS) and carboxymethyl cellulose (CMC) and characterized by FESEM, DLS, and FTIR analyses. The nanoformulation with a CMC/CS ratio of 1/2 exhibited particle sizes of 80–100 nm and demonstrated stronger antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* than the crude extract. Both the extract and its nanoformulation were more cytocompatible than dihydromyricetin, confirming their potential as safe and effective antibacterial agents.

Keywords: Acute Toxicity, *Ampelopsis cantoniensis* Planch., Antibacterial Activity, Cytocompatibility, Nanoformulations.

Introduction

Ampelopsis cantoniensis Planch. is a species of dicotyledonous plant belonging to the genus *Ampelopsis* in the grape family (Vitaceae). *A. cantoniensis Planch.* was described as vines with slightly hard stems. *A. cantoniensis* leaves are twice-doubled, with thin, brittle leaflets, low tooth margins, 4-5 pairs of secondary veins, large, round ligules.[1] *A. cantoniensis Planch.* has a sweet, light, cool taste; clears heat and detoxifies; the stem and leaves are used to brew water instead of tea; in addition, it has analgesic, anti-inflammatory, and antibacterial effects.[2] This plant can kill germs and bacteria, reduce acidity in the stomach, help peptic ulcer disease heal easily, cut pain caused by duodenal ulcer, improve digestion, and promote easy sleep. Myricetin extracted from *A. cantoniensis* leaves had a significant protective effect on LDL oxidation induced by metal ions (Cu^{2+}) or free radicals. Myricetin exhibited higher antioxidant activity than α-tocopherol in a dose-dependent manner, with the IC_{50} of 1.9 µg/mL.[3-4] In 2020, the Nguyen et al. group isolated five flavonoid compounds from the plant's ethanol extract: myricitrin, myricetin, quercetin, dihydromyricetin, and phloretin. They all exhibit strong DPPH radical scavenging activity, with SC50 values ranging from 9.42 to 35.37 µM.

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The four substances of myricetin, myricitrin, quercetin, and phloretin are good inhibitors of two enzymes α- and α-amylase glucosidase which prevent the rise in blood sugar levels after meals.[5] Liu Deyu et al also isolated Dihydromyricetin from the methanol extract of *Ampelopsis cantoniensis* Planch. The acute toxicity of this compound was assessed by intraperitoneal injection of KM.[6] In 2000, Xu Zhihong and their research team studied the nutritional and flavone components of *Ampelopsis cantoniensis* leaves and determined the protein content of 9.25% and flavon of 4.73%.[7] In 2009, the author group led by Tzu-Wei Tan studied the *in vivo* effect of *Ampelopsis cantoniensis* on leukemia WEHI-3 cells and the results showed that the treated cancer cells self-destructed.[8] Win-xing *et al.* isolated 13 compounds from the ethanol extract of the stem of *Ampelopsis cantoniensis*: resveratrol, 5,7-dihydroxycoumarin, kaempferol, dihydroluteolin, quercetin, dihydroquercetin, gallic acid, myricetin, dihydromyricetin, quercetin-3-O-α-L-rhamnoside, myricetin-3-O-α-L-rhamnoside, kaempferol-3-O-α-L-rhamnoside, epicatechin-3-O- gallate. Spectroscopic methods also determined the structures of the compounds.[9] In another study, the microwave-assisted extraction conditions to isolate dihydromyricetin from *A. cantoniensis* were optimized.[10] Despite plenty of studies on the phytochemical and biological activity of *A. cantoniensis*, its safe concentration as well as its mixture with other plants and their nanoformulations seem not to have been investigated.

Despite extensive studies on the phytochemical composition and biological activities of A. cantoniensis, there remains a lack of information on its safety concentration and on the potential enhancement of its biological properties when combined with other medicinal plants or formulated into nanocarriers.

Turmeric, or *Curcuma longa*, a species in the *Zingiberaceae* family, has long been used in traditional medicine across many Asian countries to treat inflammation, infectious diseases, and gastric, hepatic, and blood disorders.Click or tap here to enter text.[11] The most important bioactive substance of *Curcuma longa* is a polyphenol called curcumin. This substance possesses antioxidant, anti-inflammatory, antibacterial, antiviral, antitumor, and hepatoprotective activities.[12] However, poor solubility and bioavailability prevent curcumin from being used in

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clinical applications. Therefore, considerable effort has been made to encapsulate curcumin in various nanoformulations to enhance its therapeutic efficacy.[13]

Taraxacum officinale (G.H. Weber ex Wiggers), commonly called dandelion, is a perennial plant belonging to the family Asteraceae. The plant is about 40 cm tall and features by yellow to orange flowers and jagged leaves.Click or tap here to enter text.[14] It has been reported to have many medicinal properties, such as antibacterial, antiviral, anti-obesity, antidiabetic, and anti-arthritic… activities.[15] The main phytochemicals found in the flower, leaf, stem, and roots of *Taraxacum officinale* are carotenoids; flavonoids (e.g., quercetin, chrysoeriol, luteolin-7-glucoside); phenolic acids (e.g., caffeic acid, chlorogenic acid, chicoric acid); polysaccharides (e.g., inulin); sesquiterpene lactones (e.g., taraxinic acid, taraxacoside, 11β,13-dihydrolactucin, ixerin D, taraxacolide-O-β-glucopyranoside); sterols (e.g., taraxasterol, β-sitosterol, stigmasterol); triterpenes (e.g., α-amyrin).[16] Regarding the antibacterial activity of dandelion, Qian *et al.* and Wang *et al.* reported that oligosaccharides and polysaccharides extracted from the plant inhibited the growth of *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*.[17-18] Another study found that the leaf extract of dandelion exhibited strong antibacterial activity against *Staphylococcus aureus* and moderate activity against *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*.[19] Recently, a new β-branched glucomannan has been isolated from dandelion leaves and encapsulated into liposomes or solid lipid nanocarriers to improve its therapeutic activity.[20] Although plant extracts have high potential for bioactivity, their toxicity should be evaluated. A study found that *Caralluma dalzielii* N. E. Brown (Asclepiadaceae), a cactus-like plant widely used in traditional medicine for the treatment of rheumatoid arthritis, diabetes, infertility, and impotence, showed low toxicity at short-term use ($LD_{50} \geq 2000$ mg/kg) but liver toxicity at long-term use.[21] Another study evaluated the toxicity of extracts from four medicinal plants (*Senecio vernalis*, *Symphytum officinale*, *Petasites hybridus*, and *Tussilago farfara*) to *Artemia salina* and *Daphnia magna*. The results showed that all tested extracts were toxic to the tested aquatic organism. The toxicity was due to the presence of pyrrolizidine alkaloids in the extracts.[22] *Enydra fluctuans* Lour, an edible medicinal plant, was also suggested for use at a suitable dose to avoid toxicity, and caution is advised in its use in daily life. The lethal concentration (LC_{50}) on *zebrafish* of the plant extract was found to be 204.132 mg L^{-1} after 24 h. The fish exposed to 200 mg L^{-1} of *Enydra fluctuans* extracts showed severe histopathological changes.[23] Therefore, evaluating the safety and toxicity of combined plant extracts is essential prior to their practical applications. In this study, an aqueous extract of *Ampelopsis cantoniensis Planch.* combined with *Curcuma longa* and *Taraxacum officinale* (Am-CT extract) was prepared to enhance their synergistic bioactivities, particularly antibacterial properties. The toxicity of the combined extract was assessed using *zebrafish embryos* to determine its safe concentration. Furthermore, three nanoformulations of the Am-CT extract were developed using chitosan (CS) and carboxymethyl cellulose (CMC), which are biocompatible and widely used in the food and pharmaceutical industries,[24-25] to evaluate their potential in antibacterial applications. The selected extraction and nanoencapsulation methods were chosen because they are environmentally friendly, reproducible, and capable of improving both the bioavailability and safety of natural bioactive compounds, thereby aligning with the research objectives. To the best of our knowledge, this is the first study to report the toxicity assessment and nanoformulation of a mixed plant extract comprising *A. cantoniensis*, *C. longa*, and *T. officinale*, aiming to provide a safe and effective approach for antibacterial applications.

Materials and Methods

Materials

Ampelopsis cantoniensis Planch. was collected in April 2021 in Hong Ca town, Tran Yen district, Yen Bai province, Vietnam (GPS coordinates: 21°38′45″N, 104°51′20″E), including leaves and young stems. The plant species was identified and authenticated by botanists at the Institute of Ecology and Biological Resources (Vietnam Academy of Science and Technology). A voucher specimen (No. AC2021-YB01) was deposited at the institute's herbarium for future reference. Rhizomes of turmeric (*Curcuma longa* L.) and aerial parts of dandelion (*Taraxacum officinale* F.H. Wigg.) were collected in the same location and similarly authenticated. Voucher specimens for *C. longa* and *T. officinale* were assigned the numbers CL2021-YB02 and TO2021-YB03, respectively. Chitosan (CS) powder with a deacetylation degree of>75%, viscosity of < 150 cPs, was provided by S-Green Company (Ho Chi Minh City, Vietnam). Carboxymethyl cellulose (CMC) powder (food grade; Sigma-Aldrich, USA), ethanol (analytical grade, purity 99.5%; Merck, Germany), and acetic acid (analytical grade, purity 99.5%; Merck, Germany) were used without further purification. Double-distilled water was used throughout the experiments.

Am-CT extract preparation

Ampelopsis cantoniensis sample was transferred to a sealed chamber of the Memmert VO200 vacuum oven (Model VO200, Memmert GmbH, Schwabach, Germany) at CZ Pharma Company (Hanoi, Vietnam) at 40 ºC, 10 mbar, and dried until the moisture content was below 10%.

After cleaning and removing impurities, the *Curcuma longa* sample was steamed for 6-12 hours using a steam sterilizer (Model MLS-3751L, Sanyo Electric Co., Osaka, Japan). Then the steamed *Curcuma longa* was allowed to drain, cut into thin pieces, and freeze-dried (Lyovapor™ L300 (BUCHI, Labortechnik AG, Flawil, Switzerland) until the moisture content was below 10%. *Taraxacum officinale* sample including stem, root, and leaf, after preliminary treatment, was washed, chopped, and transferred to a closed chamber of a vacuum dryer (Model DZF-6050, Shanghai Yiheng Scientific Instrument Co., Shanghai, China) with pumping mode: pressure 50 mmHg, temperature 40–45°C to dry until the moisture content reached 10%.

After that, 3.50 kg of dried *A. cantoniensis* was mixed with 0.30 kg of the dried turmeric sample and 0.30 kg of the dried dandelion sample. The mixture was then ground in a hammer mill (Model DF-20, Wuyi Haina Electric Appliance Co., Zhejiang, China) using a 60-mesh sieve and stored in a sealed container.

The ground mixture was initially extracted with 15 L of water using microwave-assisted extraction (Model MARS 6, CEM Corporation, Matthews, NC, USA) at 464 W and 60 ºC in a 30 L extraction device equipped with a stirrer, a strainer, and a bottom-release valve. The first extraction ended after 3 hours. The first extract was then drained through the bottom drain valve. The second extract was performed for an additional 5 hours at 60 °C, with 12.5 L of water added to the extraction device. The second extract was drained, mixed with the first, and allowed to settle. The residue was removed from the extract by vacuum filter (Model R-300, BUCHI Labortechnik AG, Flawil, Switzerland). The extract was then concentrated under reduced pressure (50~100 mmHg) and finally free-dried by a Lyovapor™ L300 (BÜCHI Labortechnik AG, Switzerland).

Acute toxicity of the Am-CT extract on zebrafish embryos

The toxicity assessment of Am-CT extracts to determine the 50% Lethal Concentration 50 (LC50) and the 50% Effective Concentration (EC50) causing morphological effects in 50% of experimental individuals was based on the development of *zebrafish embryos* according to OECD guidelines.[26] The procedure for which is summarized as follows:

3 g of the Am-CT extract was placed in a 250 mL glass measuring flask, then approximately 200 mL of filtered water from the Geyser water filtration system was added, and the mixture was boiled at 100°C. The mixture was soaked and stirred for 30 minutes to dissolve the extract completely. Hot water was then added to reach a volume of 200 mL. The solution was stirred with a glass rod and transferred to a clean, sterilized glass flask. The concentration of the solution was calculated as 3 g of the extract in 200 mL of water, equivalent to 15 $g.L^{-1}$. The solution was then cooled to room temperature before testing on *zebrafish embryos*.

To obtain *zebrafish embryos* for the acute toxicity experiment, mature *zebrafish* were selected and transferred to matting chambers in the late afternoon and eggs were collected the following morning. The male: female ratio usually is 1:2 or 2:3 to increase the rate of spawning. Agile, fast-swimming males and well-rounded, egg-bounding females are chosen for pairing. Breeding tanks were designed to consist of two tanks stacked on top of each other; the upper tank has many holes at the bottom to prevent parents from eating their eggs. Additionally, a transparent plastic tank divider was installed in each breeding tank to separate males and females and prevent unwanted spawning at night. The previous afternoon, male and female individuals were transferred to a special breeding tank and separated by a tank divider until the next

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morning (the start of the light cycle). At the onset of light, the dividers were removed from the breeding tanks so the males and females could start mating. They were allowed to mate without disturbance for at least 30 minutes; after that, the laid eggs were returned to the system, and the collected embryos were stored.

Zebrafish embryos in the 2-8 cell stage (approximately 1 hour after fertilization) were cultured in the E3 fish embryo culture solution (5 mM NaCl; 0.17 mM KCl; 0.33 mM $CaCl_2$; 0.33 mM $MgSO_4$). The diluent was also used throughout the experiment.

The *zebrafish embryos* were washed, and discarded abnormally developed individuals before exposure in diluted concentrations from the 15 g L^{-1} stock solution. The dilution range is 1:1.5, 1:2; 1: 4; 1: 8, equivalent to 10; 7.5; 3.75; 1.875 g L^{-1}, respectively. The toxicity test plate was a 6-well plate, each well was supplemented with 4 mL of the tested solution and the exposure density was 20 individuals/well. Every 24 h, the number of dead and malformed embryos was recorded until 96 h. The test was repeated three times.

The results obtained from the replicates were used to construct the dose-response curve and calculate the LC_{50}, EC_{50}, and TI (Tetratogenic index – calculated by the ratio LC_{50}/EC_{50}) using GraphPad Prism 8.0 software. The LC_{50} index was compared with the GHS acute toxicity test standard to classify toxicity. The TI index together with EC_{50} were used to predict the likelihood of morphological abnormalities in the case of non-embryogenic substances, TI >= 3 and EC_{50} < 1000 µM indicating a high teratogenic potential in Embryo.[27]

Preparation of nanoformulations of the Am-CT extract

1% CS solution in 0.5% acid acetic was first prepared. The Am-CT extract was then dissolved in 1% CMC solution in double-distilled water. The 3 nanoformulations were obtained by adding the CMC solution to the CS solution at the volume ratio of 2:1, 1:1, and 1:2 and denoted as N1, N2, and N3, respectively. The amounts of Am-CT extract in the CMC solutions were pre-determined to obtain the same concentration of the Am-CT in the 3 formulations. The mixture was then ultrasonically vibrated for 30 minutes and magnetically stirred for 2 h. Thereafter, the mixture was centrifuged to remove some appeared precipitate and lyophilized at -45 ºC in a FreeZone 2.5 L machine (Labconco, USA).

Quantification of Dihydromyricetin in the extract

Dihydromyricetin content in the Am-CT extract was quantified by High performance liquid chromatography (HPLC) method.

An exact among of the dried Am-CT extract (27.6 mg) of was dissolved in exactly 10 mL of methanol resulting in a known-concentration solution (2.87 mg/mL). The solution was filtered through a 0.45 µm filter before being used directly for content analysis the by HPLC method.

The used HPLC system was an HPLC Agilent 1290 equipped with a DAD probe and a mass spectrometry probe. The HPLC conditions included: Agilent Proshell 120 SB-C18 column (3.0 mm x 50. mm, grain size 2.7 µm); mobile phase: acetonitrile/water – 20/80; flow rate: 0.5 mL/min; sample injection volume: 5.0 µL; detector: UV wavelength 330 nm. The dihydromyricetin standard samples were prepared in methanol at the concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.25, and 15.63 µg mL^{-1}.

Characterization of the nanoformulations

The nanoformulations were characterized by several methods. Fourier Transform Infrared (FTIR) Spectroscopy was recorded in a SHIMADZU spectrophotometer using KBr pellets in the wave number region of 400–4000 cm^{-1}. The morphology of the samples was analyzed using a field emission scanning electron microscopy (FESEM) of Hitachi S-4800. The hydrodynamic size distribution of particles in the samples was determined in a Zetasizer Nano (Malvern Instruments, UK).

Investigation of biological activity in the extract and its nanoformulations[28]

The antibacterial activities of Am-CT extract and its nanoformulations were determined by the agar diffusion method. The tested microorganism strains included Gram-positive bacteria (*Staphylococcus aureus* ATCC 13709) and Gram-negative bacteria (*Escherichia coli* ATCC 25922). Microbial culture mediums were Mueller-Hinton Agar (MHA) and Tryptic Soy Agar (TSA). Ampicillin and Cefotaxime were used as positive references.[29-30]

The bacteria that were stored at -80 ºC were subcultured to reach a concentration of 10^6 CFU/mL before experimenting. 100 µL of the activated bacteria solution was pipetted and spread evenly on the agar surface of petri dish. A 6 mm-diameter hole was punched in the petri dish. 50 µL of the sample solution (Am-CT extract and its nanoformulations) was added into the agar hole. The petri dish was covered and left for standing for 1 hour at room temperature. Then the dish was put in the incubator at 37 ºC for the bacteria to grow for 24 hours. The inhibition zone was then measured as the diameter of the sterile ring.

In vitro MTT assay of Am-CT extract and its nanoformulation in fibroblast

Normal NIH3T3 fibroblast cells (ATCC, Manassas, VA, USA) were grown in tissue culture flasks under a humidified atmosphere with % 5 CO_2 at 37 °C. To determine the cytotoxicity of Am-CT extract and its nanoformulations, cell viability was evaluated using an MTT assay. Fibroblast cells were seeded at 10^4 cells/well in 96-well plates. After 24 h, the culture medium was replaced with a fresh medium supplemented with dihydromyricetin, Am-CT extract and its nanoformulation N3 at concentrations of 500 µg/ml and were placed in an incubator for 24 h and 48 h at 37 °C with % 5 CO_2.

Then, the cells were washed with phosphate buffered saline (PBS) and 60 µl of MTT solution was added to each well followed by incubation for 4 h. Thereafter, 160 µl of DMSO solution was added to each well to dissolve violet formazan crystals. The optical density was measured at 580 nm using a microplate reader. The cell viability was calculated using the following formula (equation 1):

$$\text{Cell viability (\%)} = \frac{[\text{Abs}]\text{control} - [\text{Abs}]\text{blank}}{[\text{Abs}]\text{sample} - [\text{Abs}]\text{blank}} \times 100 \qquad (1)$$

Statistical analysis

The experiment was performed in triplicate and the data are expressed as the mean ± SD. Functions available in Microsoft Excel 2021 were applied to calculate, including mean and standard deviation.

Results and Discussion

Toxicity of the Am-CT extract on zebrafish embryos

The toxicity of the Am-CT extract was evaluated on *zebrafish embryos*. This method is considered as an alternative animal models for toxicity testing.[31] The results are summarized in Fig. 1. Am-CT extract at high concentrations affected *zebrafish embryos* as soon as 24 h of exposure (Figure 1a), most of the embryos were found to have died at a concentration of 15 g L^{-1}. In contrast, no mortality was observed during the trial at concentrations as low as 3.75 and 1.875 g L^{-1}. At concentrations of 7.5 and 10 g L^{-1}, the toxicity of the extract increased gradually with exposure time. The embryo mortality rate of 7.5 and 10 g L^{-1} extract solutions were 3% and 55% at 24 h and increased to 23% and 100 % at 96 h, respectively. The error bar depicted in the graph presenting the standard deviation shows that at low concentrations of 1.875 and 3.75 g L^{-1}, the extract could affect susceptible individuals at the concentrations. According to the GHS reference for fish toxicity testing, substances tested with an LC_{50} concentration higher than 5 g L^{-1} were classified as very safe – outside of the 5 rating.[32] Calculated by GraphPad Prism software, after 96 hours of exposure, the LC_{50} concentration of Am-CT extract reached 7.81 g L^{-1}, which is in this group of very safe substances.

Typically, *zebrafish embryos* begin to break the embryonic shell and hatch 48 hours after fertilization, and all individuals hatch after 96 hours. The number of individuals hatched at each the concentration and time was recorded and summarized in Figure 1b. It can be seen that the individuals in the control group and the 1.875 g L^{-1} concentration group hatched as expected. At a concentration of 3.75 g L^{-1}, the individuals did not hatch after 48 h, however, they still hatched after 72 h and reached a high rate (88%) after 96 h. The embryos at a concentration of 7.5 g L-1 did not hatch after 72 h, and the hatching rate at 96 h was only 17%. *Zebrafish embryos* exposed to 10 and 15 g L^{-1} extract solutions did not hatch.

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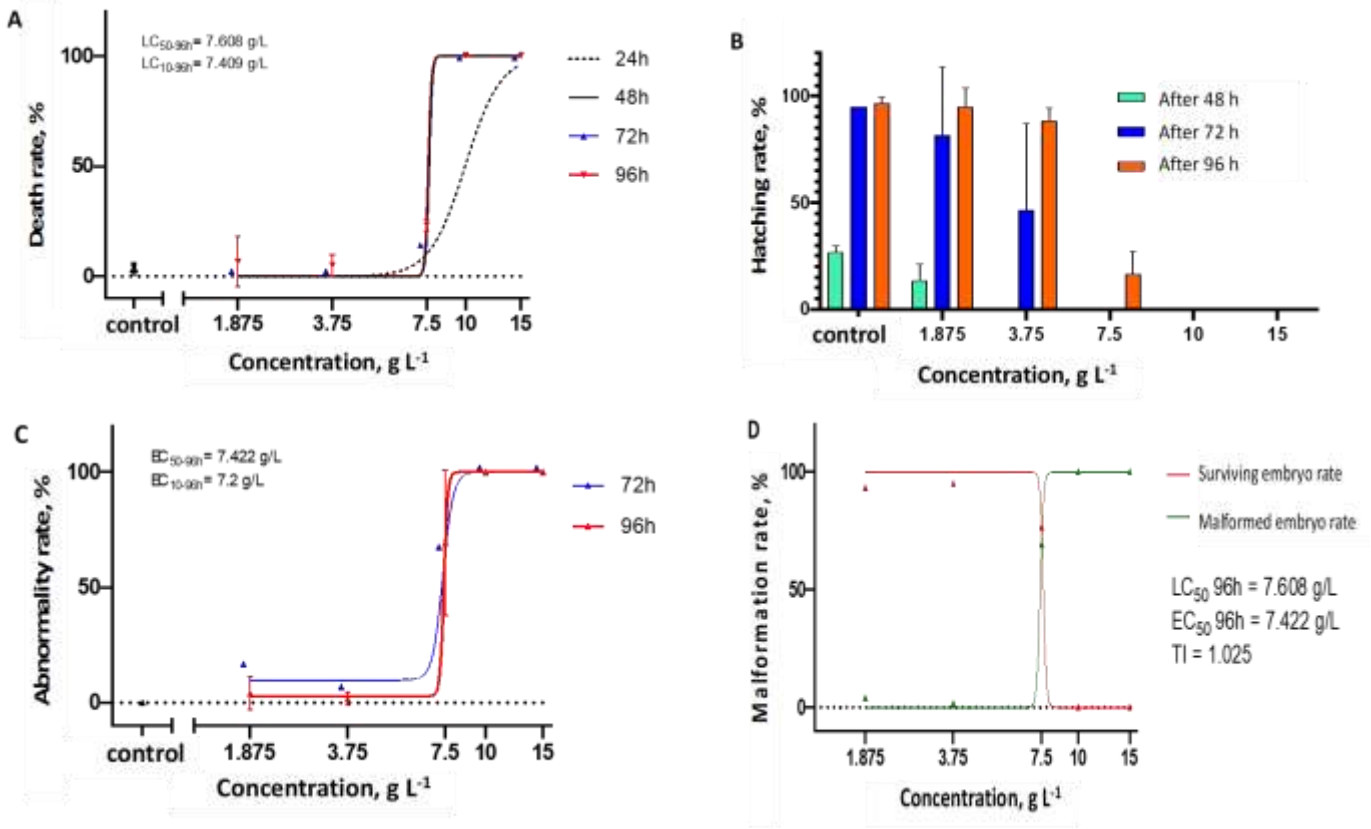

Figure 1: Toxicity of Am-CT extract on *zebrafish embryos*: (A) Mortality graph; (B) hatching rate graph; (C) Abnormality graph and (D) Graph summarizing results at 96 h exposure

Therefore, Am-CT extract caused a hatching delay at the concentration of 7.5 g L^{-1} and had little effect on the hatching ability of *zebrafish embryos* at a concentration lower than 3.75 g L^{-1}. Embryos tested at concentrations of 10 and 15 g L^{-1} died, so no hatching was observed.Figure 1c shows the abnormality rate of the embryo morphology after 72 h and 96 h exposure to Am-CT extract. The effect of the extract on *zebrafish embryos* only appears significantly at the concentration of 7.5 g L^{-1} or higher. At both concentrations 1.875 and 3.75 g L^{-1}, no abnormal embryo morphology was observed. There is little difference between the 72 h and 96 h results, so the calculated EC50 values at these time points are closely equivalent.

Figure 1D and Table 1 summarize the toxicity indices of Am-CT extract on zebrafish embryos. In Figure 1D, the green line shows the percentage of malformed embryos (the higher the line, the more abnormal the embryos). The red line indicates the number of surviving embryos (the higher the number, the more viable the embryos). The value of TI = LC_{50}/EC_{50} = 1.025 (~1) indicates that AM-CT extract had a weak ability to cause malformations in *zebrafish embryos*.[27] Most embryos at low concentrations had similar morphology (Figure 2A-D), zebrafish malformation was present at concentrations of 7.5 and 10 g L^{-1} (Figure 2E-F), indicated by the red arrow. Embryos died at concentrations of 10 and 15 g L^{-1} (Figure 2G-H). The most observed abnormality was edema (Figure 2 E-F) in which individuals present characteristic bulging areas. The abnormality may be due to a disorder in the formation of the circulatory system, leading to fluid accumulation and local edema.

Compared to the Am-RL extract, turmeric methanol extract showed much higher toxicity on *zebrafish embryos* with an LC_{50} from 55.895 to 92.415 µg/mL (or 0.059-0.0924 g L^{-1}). The embryos also died at an turmeric extract concentration of 125 µg/mL (or 0.125 g L^{-1}).[33] Another study reported that the LD_{50} of dandelion root extract on male rats ranged between 500 and 5000 mg/kg and suggested that the extract toxicity was low enough for applications. Meanwhile, there is no research on the toxicity of *A. cantoniensis*. The addition of turmeric and dandelion to *A. cantoniensis* extract did not cause toxicity for the mixed extract. The acute toxicity profile of Am-CT extract (LC_{50} = 7.81 g L^{-1}) indicates a very low embryotoxicity when compared to other botanical extracts. Similar low toxicity levels were reported for Enydra fluctuans aqueous extract with LC_{50} values in the range of several hundreds of mg L^{-1}.[34] These comparisons demonstrate that aqueous Am-CT extract is substantially less toxic than many solvent-based extracts or essential oils, possibly due to the predominance of polar compounds with lower membrane permeability.

These findings suggest that Am-CT extract has a broad safety margin for biomedical and antimicrobial applications. However, the presence of edema at higher concentrations (≥7.5 g L^{-1}) indicates mild cardiovascular or osmoregulatory disturbances that warrant further studies on oxidative stress, gene expression (e.g., cmlc2, nkx2.5), and heart rate variability to elucidate its mechanism of action. Follow-up experiments focusing on chronic exposure, nanoparticle interactions, and cytotoxicity in mammalian cells are recommended to support translational safety assessments.

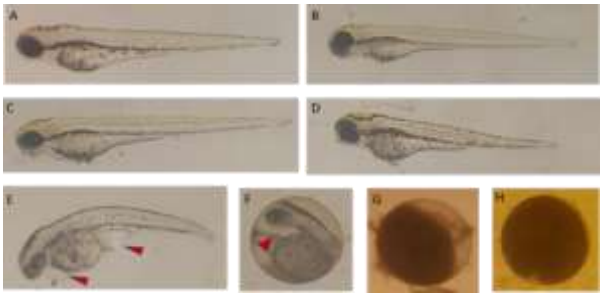

Figure 2: Effect on the morphology of Am-CT extract on *zebrafish embryos* after 96 h: (A) Control, (B) 1.875 g L^{-1}, (C) 3.75 g L^{-1}, (D-E) 7.5 g L^{-1}, (F-G) 10 (g L^{-1}); (H) 15 g L^{-1}

Dihydromyricetin content and nanoformulations of Am-CT extract

HPLC was used to determine the dihydromyricetin content of the dried Am-CT extract. Details of the HPLC results are listed in the Supporting Information. Figures S1 and S2 show UV-Vis and HR-ESI-MS spectrum of standard dihydromyricetin. Figure S3, S4 and Table S1 present the determination of calibration curve for dihydromyricetin by

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HPLC method. Figure S5 shows the HPLC chromatogram of Am-RL extract. From the chromatogram and the calibration curve, the dihydromyricetin content of the dried Am-CT extract was found to be 29.69%. The high content of dihydromyricetin may result in many bioactivities of the extract, especially the antibacterial activity.[35-36]

The FESEM images of the nanoformulations with different CS/CMC ratios are shown in Figure 3. While nanoparticles can be easily observed in the FESEM images of N1 and N3, N2 does not show separated particles. The particle size of N2 ranges from about 150 to 250 nm with some aggregations. The FESEM images of N1 and N3 show particles in the range of 60-80 and 80-120 nm for N2 and N3, respectively. Another study reported changes in nanoparticle size with varying ratios of chitosan and sodium alginate, but no clear explanation was provided.[37]

Table 1: Toxicity index of Am-CT extract on *zebrafish embryos*

	24 h	48 h	72 h	96 h
LC_{50} (g L^{-1})	9.955	7.634	7.658	7.608
EC_{50} (g L^{-1})	N/A	N/A	7.368	7.422
TI	N/A	N/A	1.039	1.025

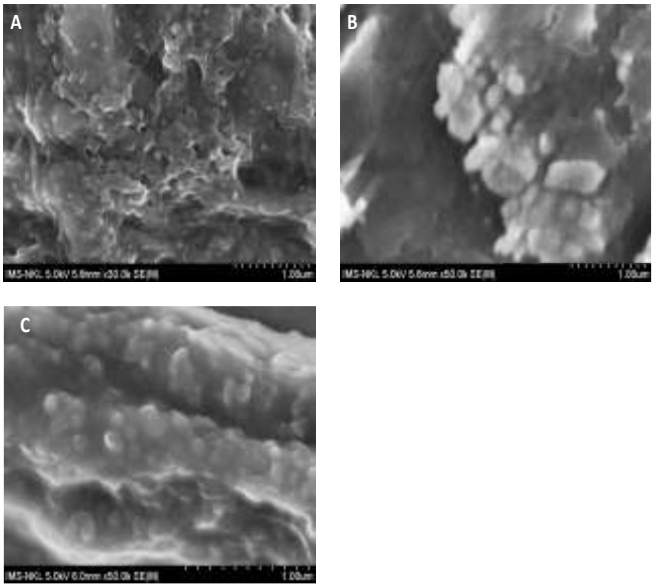


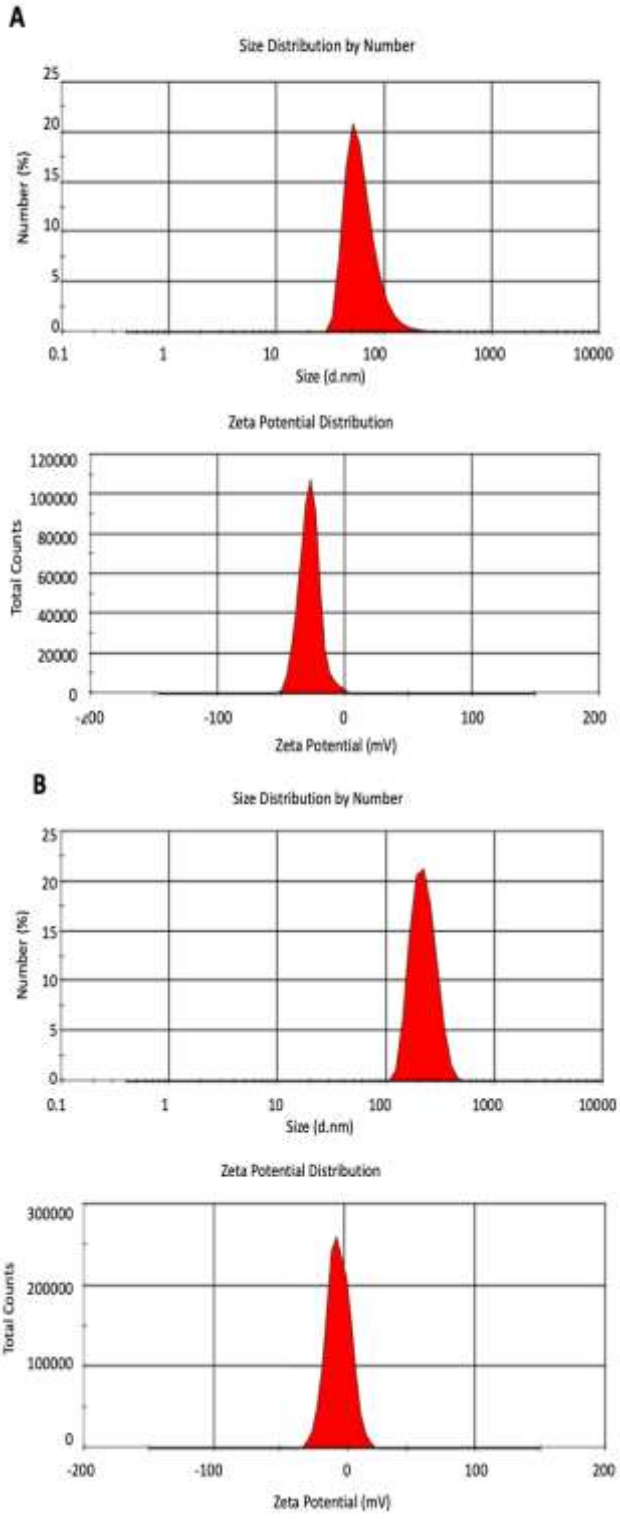
Figure 3: FESEM images of the nanoformulations: (A) N1, (B) N2, and (C) N3

The DLS measurement results are shown in Figure 4. The average hydrodynamic sizes of N1, N2, and N3 were 73.5 nm, 218.2 nm, and 89.2 nm, respectively. These results are in good agreement with those obtained from SEM images. The Zeta potential of the nanoformulations is -28.7 mV, -6.1 mV, and 21.6 mV corresponding to N1, N2, and N3. The nanoformulations' zeta potential becomes more positive as the CS content increases, due to the presence of many protonated amino groups in the sample. The low stability of N2 (zeta potential of -6.1 mV) might be the reason why it forms larger particles compared to N1 and N3.

Figure 5 presents the FTIR spectra of the CS, CMS, Am-CT extract, N1, and N3. Characteristic peaks are summarized in Table 2. The changes in peak positions confirm interactions between Am-CT, CS, and CMC in the N1 and N3 nanoformulations. The interactions can be dipole-dipole interactions and hydrogen bonds between the OH and NH groups in organic compounds of Am-CT and in CS and CMC, which have similar groups.[37] The change in the absorbance band in UV-Vis spectra of N3 compared to Am-CT (301 nm compared to 308 nm) further confirms the formation of the nanoformulation (Figure 6).

Antibacterial activities

The antibacterial activities of the samples are presented in Table 3. Gram-positive *Staphylococcus aureus* (ATCC 13709) bacteria cause pus in wounds, burns, sore throats, and purulent infections on the skin and internal organs. Gram-negative Escherichia coli (ATCC 25922) causes several gastrointestinal diseases, including gastritis, colitis, enteritis, and bacterial dysentery. At a concentration of 20 mg/mL, the Am-CT extract, N1, and N2 samples did not show an antibacterial effect on *E.coli*. The antibacterial activity of N1 and N2 against *S. aureus* is even smaller than that of Am-CT extract (inhibition zone of 13.5 mm). This can be explained by the fact that the extract was encapsulated in the polymeric matrix and may require more time to be released and exert its impact on the bacteria.



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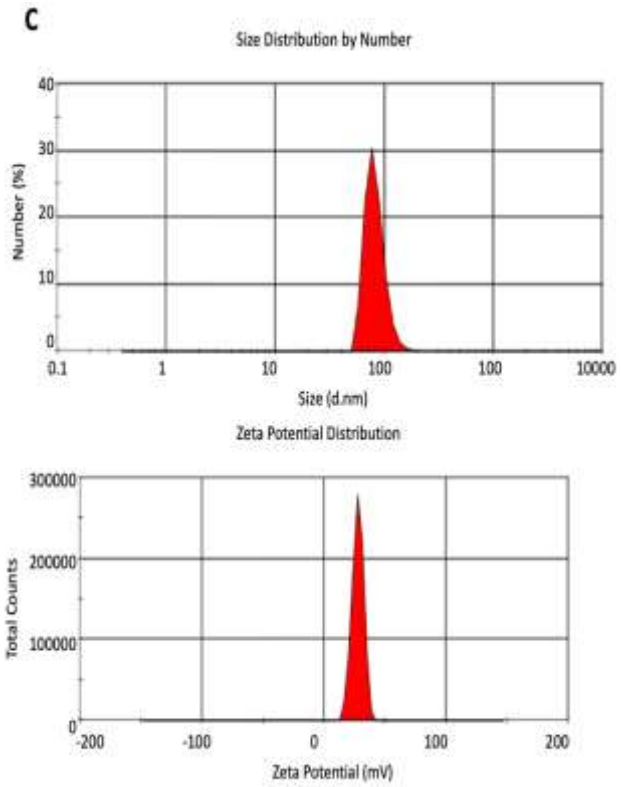

Figure 4: Size distribution and zeta potential of (a) N1, (b) N2, and (c) N3

Table 2: Characteristic peaks in FTIR spectra (cm^{-1})

	OH stretching, NH stretching	C-H stretching	C≡C or C≡N stretching	C=O stretching	N-H bending	C-O stretching
CS	3361.5	2880.34		1647.73	1586.58	1078.11 1055.90
CMC	3345.04	2885.50		1585.92		1052.64, 1021.97
Am-CT	3249.56	2937.13	2360	1607.21	1515.05	1025.54
N1	3249.20	2918.48		1582.36	1516.05	1022.37
N3	3232.94	2881.23		1596.20	1552.96	1020.30

CS – Chitosan
CMC – Carboxymethyl cellulose
Am-CT – Ampelopsis cantoniensis – Curcuma longa – Taraxacum officinale extract
N1 – Nanoformulation containing CS, CMC, and Am-CT extract at ratio 2:1
N3 – Nanoformulation containing CS, CMC, and Am-CT extract at ratio 1:2

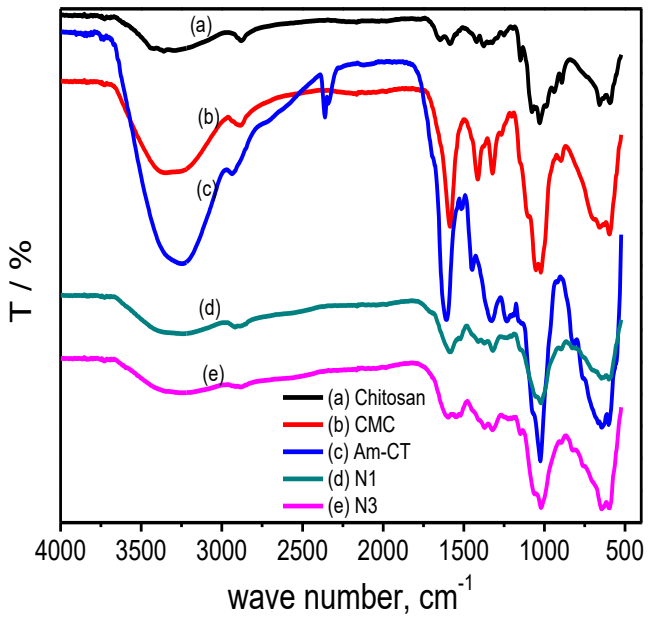

Figure 5: FTIR spectra of the samples

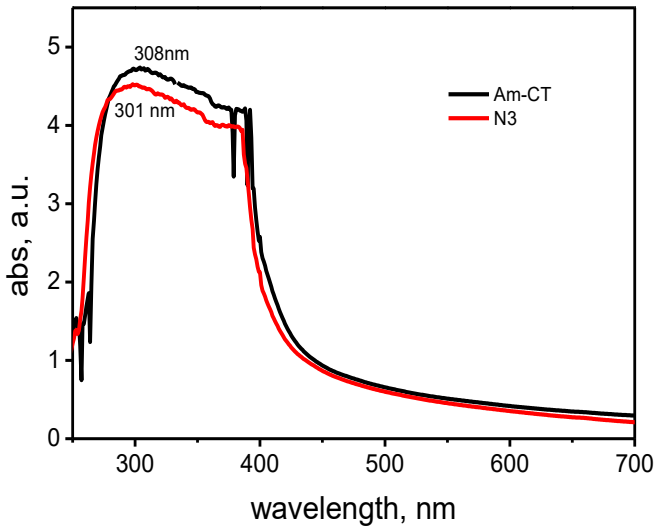

Figure 6: UV-Vis spectra of Am-CT extract and N3

A recent study found that *A. cantoniensis* ethanol extract at a 2.5 times higher concentration of 50 mg/mL showed a smaller inhibition zone against *S. aureus* of 9.5 mm.[40] This also suggested that the combination with turmeric and dandelion could improve the antibacterial activity of *A. cantoniensis* extract. N3 showed the inhibition zone against *E.coli* and *S. aureus* of 18 and 19 nm, which is much higher than that of Am-CT extract at the same concentration. The positive surface charge of N3 (zeta potential of 21.6 mV) can help the nanoparticles to bind with the negative surface of the bacteria and cause higher inhibition effect.[38] This also confirms the suitable ratio CMC/CS of 1:2 in terms of the antibacterial activity of the nanoformulations. Other reports showed similar results when using CMC and CS as the nanocarrier for in antibacterial or biomedical applications.[24-25,39] The results also suggest that N3 is potential for antibacterial applications.

Cytocompatibility of Am-CT extract and its nanoformulation

The cytocompatibility of Am-CT extract and its nanoformulation N3 of was evaluated on NIH3T3 fibroblasts. Figure 7 reveals that both Am-CT extract and N3 were highly cytocompatible with a high cell viability of around 100% at 500 µg/ml concentration. It is clearly that both the extract and its nanoformulation reduced the cytotoxicity of

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dihydromyricetin (cell viability of 56.7 and 43.3% at the same concentration after 24 and 48 h, respectively). These results could indicate the potential application of Am-CT extract and its nanoformulation as an antibacterial agent with high safety.

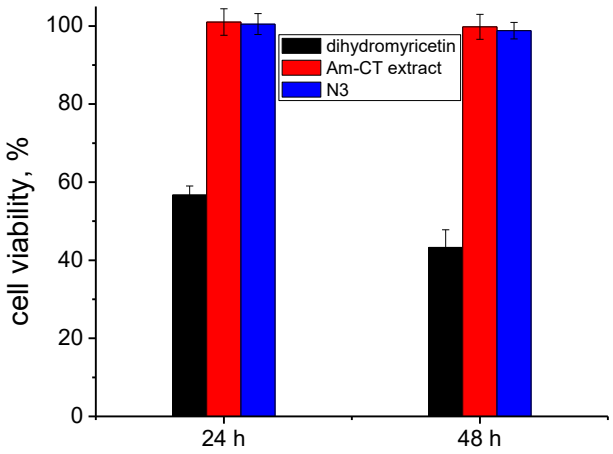

Figure 7: Cytocompatibility of the samples

Table 3: Inhibition zone (mm) of the samples

Sample	N1	N2	N3	Am-CT extract	Ampicillin	Cefotamicime
E. coli	6	6	18 ± 0.7	6	-	12 ± 1
St. aureus	11±0.3	6	19 ± 0.3	13.5 ± 0.3	26 ± 0.7	-

Conclusion

The methanol extracts of *T. bangwensis* leaf, *T. globiferus*, and *P. incana* flowers have larvicidal potential. Generally, after partitioning, their resulting fractions are more active than the methanol extracts. The *n*-hexane fraction of *T. bangwensis*, the ethyl acetate fraction of *P. incana*, and the aqueous fraction of *T. globiferus* demonstrated the highest activity. Efforts are ongoing to isolate the active compounds of these active fractions.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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