https://doi.org/10.48048/tis.2024.8412

The Development of a Novel Nanoherbal Passionfruit (*Passiflora edulis)* Leaves with Safety and Analgesic Effect

Fahleni Fahleni[1], Yesi Desmiaty[2], Rika Sari Dewi[3], Anisah Kolilah[3], Mona Sarvilia Nurul Izzah[3] and Ni Made Dwi Sandhiutami[3,*]

[1]Department of Pharmaceutical Technology Formulation, Faculty of Pharmacy, Pancasila University, Jakarta, Indonesia
[2]Department of Biology Pharmacy, Faculty of Pharmacy, Pancasila University, Jakarta, Indonesia
[3]Department of Pharmacology, Faculty of Pharmacy, Pancasila University, Jakarta, Indonesia

([*]Corresponding author's e-mail: dwisandhiutami@univpancasila.ac.id)

Received: 12 May 2024, Revised: 1 June 2024, Accepted: 28 June 2024, Published: 20 October 2024

Abstract

Passionfruit (*Passiflora edulis*) leaves are proven to contain flavonoids especially quercetin and showed a significant antinociceptive effect at acetic acid-induced wriggling. Modification of passionfruit leaf extract into nanoparticles can provide therapeutic effects precisely, quickly, and optimally. This study aimed to provide the method of preparation, and characterization of nanoherbal passionfruit leaves (NPL) and evaluate its safety profiles and analgesic effect. NPL was synthesized using ionic gelation methods, with 3 formula variations. Passionfruit leaf extract was dissolved using mixed solvents. The passionfruit leaves extract solution was suspended in Chitosan and dripped with sodium tripolyphosphate solution until nanoparticle formation. The characterization of the NPL study was evaluated by measuring the particle size, polydispersity index (PI), zeta potential, entrapment efficiency (EE), drug release, and transmission electron microscope (TEM) images. The safety profiles of NPL were then assessed in mice by acute and subchronic toxicity using OECD methods. The effectiveness of NPL as an analgesic was tested using an acetic acid test (visceral pain) in mice. We found the particle size of the NPL's best formula (F2-NPL) obtained was 187.6 nm with polydispersity index 0.626 and zeta potential +31.2 mV. The entrapment efficiency of flavonoid content in NPL was 50.9 % and the flavonoid release for 3 h with 3 replications were 22.91, 27.88 and 25.27 % respectively. The morphology of the particles by TEM showed that the nanoparticles were circular shape. Safety evaluation in mice resulted in the LD_{50} of NPL being greater than 5000 mg/kg in the acute toxicity study and had no adverse effects on blood, liver, and renal profiles in the subchronic toxicity study. The NPL-200 group had better effectiveness as an analgesic (84.87 %) compared to NPL-50 (37.80 %) and NPL-100 (69.15 %). This finding indicates that the NPL is a safe and effective formula for enhancing analgesic effects. Thus, enables its applications for the treatment of various diseases related to analgesics and inflammation such as osteoarthritis.

Keywords: Passionfruit leaves, Nanoherbal, Acute toxicity, Subchronic toxicity, Analgesic

Introduction

Long before there were health services, traditional medicine was employed as an alternative kind of treatment [1]. In Indonesia, one of the plants that is efficacious and developed as a medicine is passionfruit leaves (*Passiflora edulis*). Passionfruit leaves from the Passifloraceae family have been shown to offer a number of therapeutic uses and advantages, including analgesic, anti-inflammatory, antidiabetic, anxiolytic,

anticancer, antidepressant, antioxidant, and anticonvulsant properties [2]. Based on phytochemical research, passionfruit leaves (*Passiflora edulis*) are proven to contain polyphenols, alkaloids, flavonoids, triterpenoids, carotenoids, and glycosides. Carotenoids, polyphenols, and vitamin C compounds that have effects as antioxidants [3,4]. Evidence from earlier studies demonstrated that extract from *Passiflora edulis* leaves, at doses of 100, 200 and 400 mg/kg BW, significantly reduced wriggling caused by acetic acid and acute inflammation in the animal-tested models [5].

The development of traditional medicine continues to be improved, to obtain the availability of safe, high-quality, efficacious traditional medicines that are scientifically tested and can be widely utilized both for self-treatment by the community and in health services [6,7]. It is known that the most widely reported flavonoid derivative contained in *P. edulis* leaves is quercetin [4]. Quercetin has poor bioavailability, solubility, and stability, reducing its therapeutic effect [8,9]. In drug research, poor water solubility continues to be a significant challenge that requires constant innovation to address. By creating nanoparticles, adjustments can be made to natural products including quercetin in flavonoids in order to maximize their activity and boost their bioavailability, solubility, and capacity to be swiftly absorbed in the body to deliver the best possible therapeutic effects [10,11]. Modification of passionfruit leaf extract into nanoparticle form (1 - 1000 nm) is expected to increase the bioavailability of passionfruit leaves in the body, increase absorption, and release active substances in a short time so that they can reach the target organs and provide therapeutic effects precisely, quickly, and optimal [12,13]. Particle size reduction to the nanoscale increases the drug's or active herbal compound's bioavailability in target organs and increases the particle's capacity to permeate colloidal intercellular gaps [14,15]. However, the structural elements and physical properties that control solubility also have an impact on pharmacokinetics, potency, and toxicity [16]. Increasing the amount of drug in the blood during systemic delivery will increase the risk of side effects until the toxic level is reached [17].

While drug-based research on nanotechnology as drug delivery systems has been around for a while, pharmacology has only lately begun to explore nanotoxicology - the toxicity of nanomaterials. Nanocarrier systems' toxicity extends to molecular, physiological, and physicochemical issues. Direct contact between nanoparticles and DNA molecules or associated proteins may result in physical harm to the genetic material if the particle translocates into the nucleus [18-20]. Although problems with conventional therapy are addressed by nanoparticles, there are still unresolved issues such as toxicity and side effects that should be carefully considered before using them in biological systems.

In an effort to meet the requirements of standardized herbal medicine in Indonesia, passionfruit leaf nanoherbal must go through several tests. One of them is toxicity or safety testing. In developing passionfruit leaf nanoherbal using the ionic gelation method, several solvents were used as well as chitosan and tripolyphosphate sodium croslinkers. It is known that no chemical substance can be said to be completely safe, because every chemical substance is toxic at certain dose levels [21,22]. Until now there is no data that supports safety information regarding passionfruit leaf nanoherbal preparations. Therefore, in order for the passionfruit leaf nanoherbal preparation to be used safely, it is necessary to carry out a toxicity test on the preparation. It is important to study the toxicity profile further to obtain safe traditional medicines. The safety of using traditional medicines is related to toxicological data and undesirable effects on traditional medicines based on internationally accepted standards for testing product safety, namely OECD 425 for acute toxicity testing and OECD 407 for subchronic toxicity testing methods [23-25].

The objective of this study was to provide the method of preparation, and characterization of nanoherbal passionfruit leaves (NPL) and evaluate their safety profiles. This research also assessed the development of a nanoparticle formulation of passion fruit leaf extract that can provide better pain inhibition effectiveness in mice induced by glacial acetic acid according to Sigmund's method.

Materials and methods

Materials

Passionfruit leaves (*Passiflora edulis*) obtained from the Center for Spices and Medicinal Plants (BALITTRO) with identification number: 992/UN2.F3.11/PDP.02.00/2023. *Deutschland Denken Yoken* (DDY) mice; Quercetin standard and Chitosan (Sigma, Aldrich); Acetic Acid (glacial) and DMSO from Merck, KGaA, Frankfurter, Germany; Propylenglycol (SK picglobal Co., Ltd, Seoul, Korea); Tween 80 (Micromaster Laboratories Pvt.Ltd, India); CMC Na, Aqua Destilata, Maltodextrin, Na EDTA 10 %, and tripolyphosphate sodium were purchased from Brataco, Indonesia; Diclofenac sodium (Novell, Indonesia), Clinical biochemical examination kit Albumin, AST (*Aspartate aminotransferase*), ALT (*Alanine aminotransferase*), BUN (*Blood Urea Nitrogen*), and Creatinine from Reiged Diagnostics.

Preparation of aqueous extract of *P. edulis* leaves

One hundred g of passionfruit (*Passiflora edulis*) leaves simplisia powder was extracted with 1000 mL of water using the decoction method (1:10) at 100 °C for 35 min [26]. The decoction was filtered and the filtrate was collected in an Erlenmeyer. The filtrate obtained was put into a dehydrator at 40 °C until a thick extract was formed.

Determination of total flavonoid in extract of *P. edulis* leaves

Determination of total flavonoid in the extract of *P. edulis* leaves was started with optimization of the analytical method (optimum wavelength and operating time) and preparation of the standard curve of quercetin. The standard curve concentrations used were 30, 35, 40, 45, 50, and 60 ppm. 500 mg of extract of *P. edulis* leaves was dissolved in 96 % ethanol. 1 mL of the test sample was taken and added with 0.2 mL of 1 M sodium acetate, 0.2 mL of 10 % aluminum chloride ($AlCl_3$), 3 mL of 96 % ethanol, and 5.6 mL of distilled water, stirred until homogeneous. Then incubated using room temperature in a closed and dark room according to the stable time obtained from the operating time. Furthermore, the absorbance of the test sample was measured using UV-visible 1900-UV (Shimadzu, Japan) at a wavelength of 440 nm, and then the concentration was calculated using linear regression of the standard curve that has been made [27].

Preparation, evaluation, and characterization of nanoherbal passionfruit (*P. edulis*) leaves

Nanoherbal passionfruit (*P. edulis*) leaves (NPL) were made at the Pharmaceutical Technology Formulation, Faculty of Pharmacy, Pancasila University. Prepared 0.2 % chitosan in 1 % acetic acid and 0.1 % sodium tripolyphosphate. The thick extract of passion fruit leaves was dissolved with a mixture of solvents (consisting of 5 mL aquabidest, 5 mL DMSO, 10 mL propylene glycol, and 5 mL tween 80) to obtain good solubility. The preparation of nanoherbal was done by mixing 0.2 % chitosan solution with passion fruit leaf thick extract solution. The mixture was stirred with a magnetic stirrer at 500 rpm for 30 min. Sodium tripolyphosphate 0.1 % was added with the ratio of chitosan and sodium tripolyphosphate 5:1, at the rate of 1 drop per 3 s through a burette and with a magnetic stirrer at 500 rpm for 1 h until a homogeneous nanoherbal solution was formed. We designed 3 nanoherbal formulas as shown in **Table 1**.

Table 1 Nanoherbal formulas design.

Materials	Formula (%b/v)		
	F1-NPL	F2-NPL	F3-NPL
P. edulis leaves extract	0.25	0.50	0.75
Chitosan 0.2 %	0.13	0.13	0.13
Sodium tripolyphosphate 0.1 %	0.01	0.01	0.01

After that, we monitored the color, turbidity, and sedimentation of the nanoherbal passionfruit leaves (NPL) for thirty days. The average and size distribution, polydispersity index (PI), zeta potential, entrapment efficiency (EE), release of flavonoid content from NPL, and transmission electron microscopy (TEM) pictures of the NPL were obtained. Particle size and distribution are measured using the Particle Size Analyze (PSA) instrument (Malvern Zetasizer Nano, Worcestershire, UK). The Zeta potential of nanoparticles was used to quantify particle charge using a zeta sizer (Malvern Zetasizer Nano, Worcestershire, UK). Transmission electron microscopy (Hitachi HT7800, Tokyo, Japan) used to observe their surface morphology [28].

Determine the entrapment efficiency (EE) of NPL

Entrapment Efficiency of flavonoid compound from passionfruit leaves in chitosan- tripolyphosphate sodium complex was calculated. A total of 10 mL of nanoparticle suspension formula was precipitated by centrifugation at 14000 rpm for 30 min. Each supernatant obtained was taken as 1 mL then added 0.2 mL of 10 % aluminum chloride, 0.2 mL of 1 M sodium acetate, 3 mL of 96 % ethanol and 5.6 mL of distilled water and homogenized. The absorbance of each solution was then measured on a 1900-UV UV-visible spectrophotometer (Shimadzu, Japan) at a wavelength of 440 nm. The absorbance was obtained to calculate the concentration of total flavonoid absorbed using the calibration curve equation [28].

Calculation of EE:

$$\text{Entrapment Efficiency (EE)} = \frac{C_0 - C_1}{C_0}\ x\ 100\ \% \quad (1)$$

C_0 = the weight of the active compound at first (mg)
C_1 = free active compound weight (mg)

Release of flavonoid from NPL

A volume of dissolution medium, namely 0.2 M phosphate-buffered saline pH 6.8 was put into a 500 mL container. A total of 50 mL of NPL was put into a dialysis membrane bag which was placed into the container containing the medium at a speed of 50 rpm while simultaneously starting a stopwatch. Samples were taken at times (in min) of 15, 30, 45, 60, 90, 120, 150 and 180 by 5.0 mL in the area midway between the surface of the dissolving medium and the top of the container and not less than 1 cm from the surface of the container wall, then dissolved 5.0 mL of 96 % ethanol. Each sample from various times was measured to determine of flavonoid method using the method as previously mentioned with quercetin as a reference [27,28].

Experimental animals

Every experimental protocol was carried out from 8 AM to 4 PM during the day. The Pharmacology Laboratory, Faculty of Pharmacy, Pancasila University, Srengseng Sawah Jagakarsa, South Jakarta, is the site of this experimental study on the acute toxicity, subchronic toxicity, and analgesic effect of NPL. The Ethics Committee of the Faculty of Medicine, University of Indonesia-Cipto Mangunkusumo Hospital, approved the study. Mice were utilized as test subjects, and they underwent a week of acclimation beforehand. Every day, the experimental room's condition is likewise maintained. The temperature and humidity levels are set at 24 ± 2 °C and 50 - 60 %, respectively. The mice's lighting was changed in accordance with the guidelines, using a standard 12-h light/dark cycle.

Acute and subchronic (repeated dose 28-day oral) toxicity studies.

The study was carried out under the KET-104/UN2.F1/ETIK/PPM.00.02/2024 ethical approval number. In order to ascertain the LD_{50} value and identify any potential hazardous effects from the acute administration of NPL, an acute toxicity test was carried out. A single dosage of the test dose was given p.o. The following Organization for Economic Cooperation and Development (OECD 425) (Up-and-Down Procedure) conducted acute toxicity testing: A dose of 175 mg/kgBB NPL was administered in the first test. The following animal receives a higher dose if the mice survive, and a lower dose if they pass away. The experiment was terminated if 3 deaths occurred at the same 4 test concentrations, or if 3 living animals were found at the test's upper limit of 5000 mg/kg BW. The following observation time is conducted in 14 days in accordance with OECD 425. To find out how administering test preparations affected the activity of the test animals and any toxicity signs that developed in them, test animals were observed. Toxic symptoms, including tremors, convulsions, salivation, diarrhea, allergies, coma, skin reactions, eyes, righting reflex, and motor activity, were noted along with any deaths. The maximum likelihood method was applied statistically to get the LD_{50} values, which were placed into AOT425StatPgm *software* [25,29].

The Subchronic Toxicity Test conducted in this study refers to the OECD 407 method: Acute Oral Toxicity - Repeated Dose 28-Day Oral Toxicity Study in Rodents which lasted for 28 days using 5 groups, namely the normal control group, the dose of 50, 100, and 200 mg/kg BW and the satellite group (received a dose of 200 mg/kgBW, for observation of late toxicity signs on day-42). Forty test animals were grouped randomly. Each dose group used 5 female mice and 5 male mice. NPL was orally administered by gavage to mice once a day for 28 consecutive days. The general appearance of the mice was observed daily for any apparent signs or symptoms of toxicity during the experiment. The weight of the mice and food consumption were also monitored. On day 28, the experimental animals were fasted overnight. Their blood samples were collected. Blood was used to detect hematological analysis using Auto Hematology Analyzer Nihon Kohden MEK 6450 K at the Animal Study Centre Laboratory, Bogor. Clinical biochemical examinations were performed for the parameters AST (Aspartate aminotransferase) or SGOT (Serum glutamic axaloacetic transaminase), ALT (Alanine aminotransferase) or SGPT (Serum glutamic pyruvic transaminase), BUN (Blood Urea Nitrogen), Creatinine, Albumin by following the procedures contained in the testing kit using the Microlab 300 & 300 LX EliTech Clinical System tool [24,25].

Following the blood collection on day 28, the experimental animals underwent harvesting liver and kidney, perfusion using 10 % neutral buffered formaldehyde (NBF) fixation. After being fixated, the samples were dehydrated in graded anhydrous ethanol, embedded in paraffin. Hematoxylin and Eosin (H&E) were applied to the ~5 mm thick fine tissue sections, which were then viewed with an Olympus BX50 light microscope.

***In vivo* analgesik study with acetic acid test (visceral pain)**.

This experimental study was conducted after obtaining ethical approval with number: KET-146/UN2.F1/ETIK/PPM.00.02/2024. The analgesic effect was screened acetic acid-induced writhing test. 25 DDY mice were split into 5 groups at random (n = 5). Before being stimulated with 3 % acetic acid intraperitoneally, mice were pre-treated with nanoparticle base, Diclofenac sodium 0.39 mg/20gBW, or NPL (50, 100, and 200 mg/kgBW) for 30 min. After 5 - 10 min, there was an onset of abdominal writhing. Mice that get an intraperitoneal injection of 3 % acetic acid writhe in their abdomens, stretching, twisting to one side, retracting the abdomen, and becoming opisthotonus so that the rodent's stomach contacts the ground. The mice were observed to exhibit an abdominal writhing reaction. The number of writhing episodes was tallied every 5 min for 1 h [5,30,31].

To calculate the percentage of analgesic activity and the percentage of inhibition compared to the positive control, the AUC (Area Under Curve) value was calculated in each group, followed by calculating the percentage of writhing inhibition in each test group.

$$AUC = \frac{Number\ of\ abdominal\ writhing\ \{(\text{in min}\ (\text{n}-1)+\text{min}\ (\text{n})\}\{(min(n)-min\ (n-1)\}}{2} \quad (2)$$

$$\%\ Analgesic\ activity = 1 - \frac{AUC\ test\ group}{AUC\ negative\ group}\ x\ 100\% \quad (3)$$

$$\%\ Analgesic\ effectivity = \frac{\%\ analgesic\ activity\ test\ group}{\%\ analgesic\ positive\ control\ group}\ x\ 100\% \quad (4)$$

Statistical analysis

The data are displayed as the mean ± SD for analgesic evaluation and subchronic toxicity. The data were analyzed using a one-way ANOVA, with post hoc multiple comparisons using the least significant difference (LSD) method. $p < 0.05$ was considered significant in every instance.

Results and discussion

Determination of total flavonoid in extract of *P. edulis* leaves

In the determination of total flavonoid in *P. edulis* leaves extract, quercetin was used as a standard compound. The maximum wavelength obtained was 440 nm with an operating time of 45 min and the quercetin standard curve equation is y = 0.0537 + 0.0064x. The measurement of flavonoid content in P. edulis leaves extract was done on the standard curve equation and obtained a level of 5.51 %.

Stability of nanoherbal passionfruit leaves (NPL) nanoparticles

The color, turbidity, and sediment stability of NPL were tracked for 5 days following formulation. We discovered that the turbidity remained constant, the color did not change on the 30th day, and there were no sample deposits in the vial's bottom (**Table 2**).

Table 2 NPL physical stability test results on the 30th day.

Formula	Parameter	
	Color	**Turbidity**
F1-NPL	Brownish yellow	Stable
F2-NPL	Light brown	Stable
F3-NPL	Brown	Stable

Note: F (1-3): Formula (1-3); NPL: Nanoherbal Passionfruit Leaves

Particle size, polydispersity index (PI), and zeta potential

The results of the examination of particle size, polydispersity index, and zeta potential can be seen in **Table 3** and **Figure 1**. Based on the particle size examination, the smallest particle size in F2-NPL was 187.6 nm, PI 0.626 with a zeta potential of +31.2.

Table 3 Characterisation of nano herbal: Particle size, Polydispersity Index (PI) and Zeta potential.

Formula	Particle Size (nm)	Polydispersity Index	Zeta potential
F1-NPL	201.1	0.439	+27.3
F2-NPL	187.6	0.626	+31.2
F3-NPL	275.4	0.624	+29.4

F (1-3): Formula (1-3); NPL: Nanoherbal Passionfruit Leaves

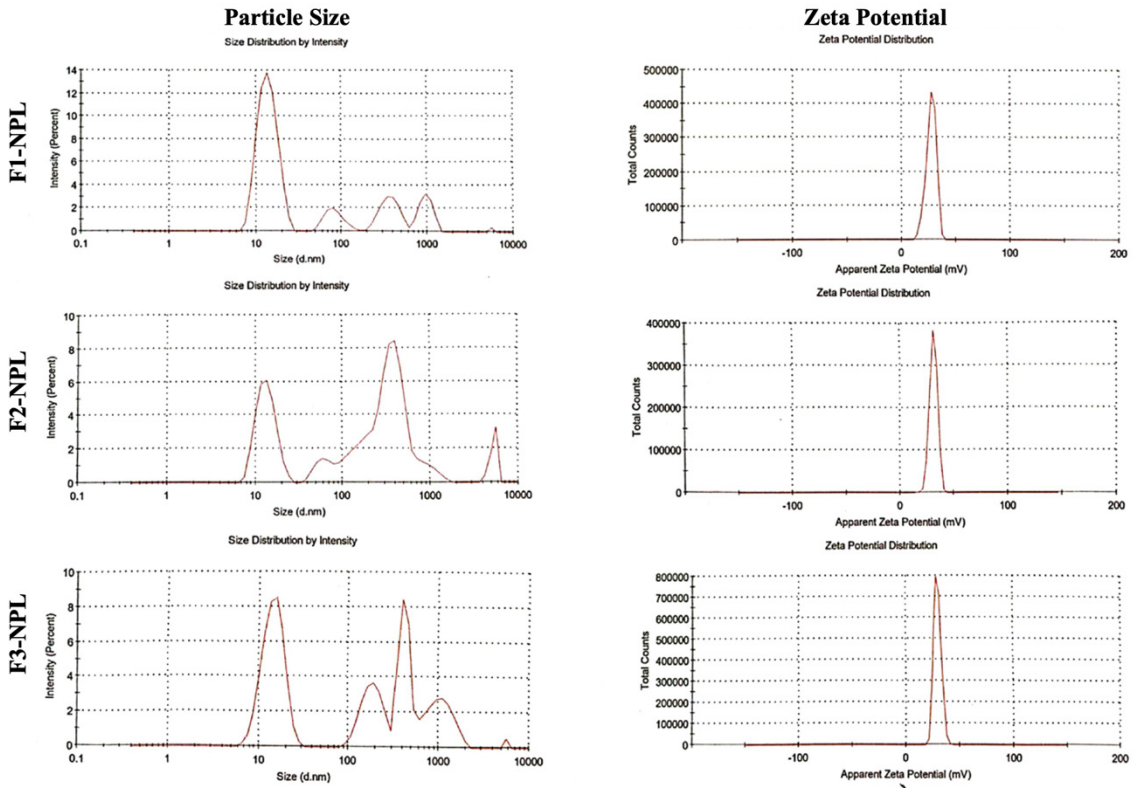

Figure 1 Particle size distribution and Zeta potential of F1-NPL, F2-NPL, and F3-NPL fulfills the criteria for nanoparticles. F (1-3): Formula (1-3); NPL: Nanoherbal passionfruit leaves.

Passionfruit leaves rich of quercetin, an important flavonol [5]. Flavonoids like quercetin have been shown to have a number of medicinal benefits, including anti-inflammatory, analgesic, anti-diabetic, anti-hypertensive, and anti-carcinogenic effects. However, because of quercetin's low water solubility, quick clearance, metabolism, enzymatic breakdown, molecular stability, and absorption properties, its bioavailability is often low. Poor bioavailability consequently hinders clinical use [16]. Our current formulation of NPL resulted in satisfactory particle size, i.e., 187.6 nm. Our result shows that the passionfruit leaves extract nanoparticles prepared, met the requirements of having a particle size of 10 - 1,000 nm. The long-lasting and target-specific nanoparticles have the potential to be used as carriers in controlled-release drug delivery schemes [32]. By formulating and reducing the particle size, the solubility of a substance or drug can be increased [33]. The absorption of compound can increase so that the plasma concentrations are increased by nanoparticles [28].

Up to thirty days following formulation, NPL at the final formulation demonstrated outstanding stability in our tests. The choice of polymers and cross-linkers has an impact on the production of nanoparticles by the ionic gelation method. Because of its biodegradability, bioadhesivity, bioactivity, and low degree of immunological reactions, chitosan is regarded as a highly biocompatible biopolymer with a

wide range of applications [34]. The best cross-linker to create nanoparticles is sodium tripolyphosphate or cross-linking agents. Sodium tripolyphosphate is a small ion that has 3 negative charges across the pH range that is physiologically acceptable [35]. When tripolyphosphate sodium anions are added, it can appropriately create a cross-link that facilitates ionic molecular interactions between chitosan ions. Since our NPL PDI of 0.626 met the criterion for particle size distribution, we may conclude that the dispersion of the passionfruit extract was homogeneous. The particle diameter index, or PDI, measures the particle's radius uniform distribution's width. A decent PDI value is between 0 and 1, with the distribution being more uniform closer to 0 [36]. There are 2 categories for the polydispersity index: monodispersity and polydispersity. In contrast to polydisperse, which has a value of > 0.7 and is characterized by particles that readily form aggregates, monodisperse has a range of 0.01 - 0.7 and is characterized by a good degree of particle uniformity, making it more stable than polydisperse. Thus, a polydispersity index value of less than 0.7 is considered desirable [37]. Based on the results obtained, show that the NPL polydispersity index in the 3 formulas meets the requirements for being included in the monodispersion category, so it is said that the size of the nanoparticles produced is uniform.

The potential zeta of NPL is +31.2. They indicate the uniformity of positively charged particles in the suspension of nanoparticles made so that the possibility of aggregation between particles decreases, and the nanoparticle becomes stable. These zeta potential NPL results are quite far from the value of 0. A good potential zeta value is a value that keeps away from the number 0, both positive and negative. If the potential zeta value approaches 0, it can allow aggregation to occur, which makes an unstable nanosuspension. Different charges with the same number of nanoparticles might lead to particle aggregation. Therefore, a higher charge will result in a more stable particle due to the higher resistance between particles. Because the positively charged nanoparticles interact electrostatically with the negatively charged mucin, they will quickly pass through the mucus layer. Positively charged nanoparticles had uptake rate constants that were about 20 times higher than those of negatively charged nanoparticles [38].

Examination of shape and morphology of *P. edulis* leaves nanoparticles (F2-NPL)

The TEM examination results are shown in **Figure 2**. The particle's round shape is evident from the TEM inspection, which provided the morphological results for this investigation. The circular shape indicates that NPL is more easily passed across the membrane. Circular shape nanoparticles are the most appropriate shape for drug delivery applications out of all of them. A nanoparticle's form is just as important to its biological function and reactivity as its size. Generally, circular-shaped nanoparticles are easier to endocytose [39].

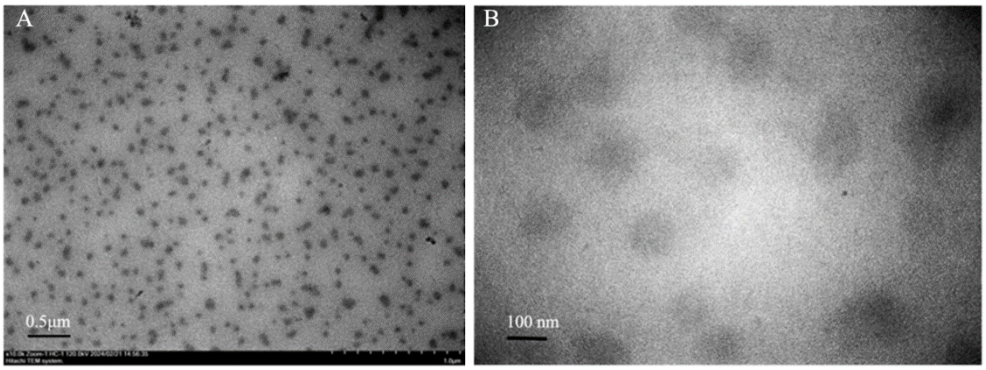

Figure 2 Transmission Electron Microscopy (TEM) Nanoherbal Passionfruit Leaves. (A) photomicrograph with scale bar = 0.5 µm and (B) Scale bar = 100 nm.

Entrapment efficiency (EE) of the NPL nanoparticles

The EE measurement of NPL nanoparticles resulted in a relatively high yield, which was in F1-NPL 59.75 %, F2-NPL 50.96 %, and F3-NPL 36.17 %. The difference in the entrapment efficiency of each formula was due to differences in the concentration of passionfruit leaf extract. This is consistent with a recent study that shows that as extract concentration increases, entrapment effectiveness would also decrease due to the limited mobility of high molecular weights [40].

Entrapment efficiency is used to determine the amount of flavonoid compound in *P. edulis* leaf extract that has been successfully absorbed in nanoparticles. High entrapment efficiency is very beneficial because it can transport enough drugs to the target organs and increase drug contact time [41]. The protonated amine chitosan group is responsible for the remarkable entrapment efficiency. Consequently, raising chitosan's ability to bind quercetin leads to flavonoid absorption in the polymer matrix and high entrapment efficiency. The ability of chitosan and tripolyphosphate sodium to shield the active ingredient from outside factors that could harm it increases with the entrapment efficiency value, which in turn increases the active substance's bioavailability [42]. Because of its cationic polysaccharide nature, chitosan is not a good choice for entrapping hydrophobic medications. The solubilization and permeability of hydrophobic medicines can be improved by chitosan-based nanoparticles, which also boost loading capacity and enhance bioavailability [43].

Release of flavonoid from NPL nanoparticles

The release test of flavonoid from passionfruit leaf extract nanoparticles was conducted to determine the amount of the flavonoid released by its carrier into a medium similar to body fluids. The release test was carried out using phosphate-buffered saline pH 6.8 because the pH corresponds to the pH of the intestine, which was carried out within 3 h. From the results of the study, the release test of passionfruit leaves nanoparticles containing quercetin and others flavonoid, there was a continuous increase in the percentage dissolved proportional to time. The release test results obtained for 3 h with the percentage of drug release in the first, second, and third replications were 22.91, 27.88 and 25.27 %, respectively. The results obtained in this study are similar to other studies that obtained quercetin nanoparticle release results of 25 % at the 3rd h [44].

NPL safety through acute toxicity test

The development of passionfruit leaf nanoparticles is a novel way to prepare passionfruit leaves, which can have greater effects at lower doses than extract preparations, despite the fact that extract from passionfruit leaves has a range of documented pharmacological effects, particularly analgesic and anti-inflammatory effects. Naturally, information regarding the possible toxicity and negative effects of this product has not yet been included in its development. As a result, the OECD Guideline 425 protocol was used in this investigation to evaluate the acute oral toxicity of NPL. The assessment of probable target organs of toxicity, toxic doses (LD_{50}) of NPL, and acceptable doses for extended toxicity (sub-acute and sub-chronic) as well as future pharmacological research necessitate the use of acute oral toxicity tests [24].

In this study, an Acute toxicity study at a limited oral dose of 5000 mg/kgBW revealed normal behavior and demonstrated no death or lethargy and no signs of toxic effects in any behavioral patterns up to 14 days. The LD50 of NPL is greater than 5000 mg/kgBW, orally, and it seems safe and non-toxic.

In this study, the LD_{50} NPL values are greater than 5000 mg/kgBW. This result is in the practically non-toxic category. Within 24 h to 14 days at the smallest dose, namely 175 mg/kgBW, and the largest dose, namely 5000 mg/kgBW, there was no death response in the test animals. The results of determining

the LD_{50} value using AOT425StatPgm software according to OECD guidelines 425, can be seen in **Table 4**.

Table 4 LD_{50} Nanoherbal *P.edulis* leaves.

Dose (mg/kgBW)	Short Term Response	Long Term Response
175	O	O
550	O	O
1750	O	O
5000	O	O
5000	O	O
5000	O	O

Description: O = Live animals, X = Dead animals

In determining the LD_{50} value, the administration of a dose of 175 mg/kgBW did not find any deaths in short-term observations or long-term observations. Doses of 550, 1750, and 5000 mg/kgBW also showed the same results, namely no deaths were found in short-term observations or long-term observations. The results of behavioral observations of test animals at all doses which have been carried out at the 30^{th} min up to 14 days showed no tremors, excessive salivation, allergies, diarrhea, convulsions, eye disorders, coma, skin reactions, righting reflex and changes in motor activity in test animals. The test animal was able to turn over and was able to hang up quickly and the eyes were clear and the skin and the test animal were normal and healthy. There were no symptoms of salivation, diarrhea, tremors, convulsions or coma. In addition, there was no decrease in motor activity in the test animals.

Nanoherbal *P. edulis* leaves (NPL) are classified as practically non-toxic nanoparticles. Nano-sized passion fruit leaves can easily pass through the body's lipid membrane due to their small size. This also allows them to avoid several barriers, including the pH of gastric acid, liver metabolism, and increased blood circulation of drugs. NPL preparations are practically non-toxic and safe for the body. Acute toxicity indicates that NPL at the limited test dose of 5000 mg/kgBW was safe to take orally.

NPL safety through subchronic toxicity tests (repeated dose 28-day oral toxicity study)

Evaluation of changes in mice body weight

Observation of mice body weight is one of the criteria of subchronic toxicity test observation (**Figure 3**) to evaluate the pathology conditions of test animals that may be caused by the harmful effects of the test substance. Based on **Figure 3**, the body weight of male and female mice from before treatment to the 28th day of treatment there was an increase in body weight in the normal control group, satellite group, and all test groups. These results indicate that the administration of NPL for 28 days in male and female mice is not affected by the dose level, there are no toxicity symptoms that cause loss of appetite.

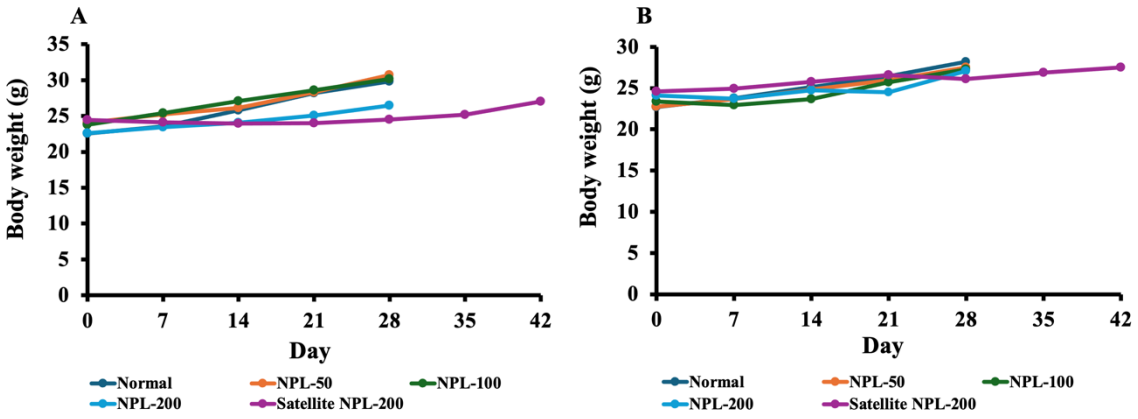

Figure 3 Body weight gain of male mice (A) and female mice (B); Data are expressed as the mean; N = 5 mice per group. NPL: Nanoherbal Passionfruit Leaves; Satellite NPL-200: group received a dose of 200 mg/kgBW, for observation of late toxicity signs on day 42.

Hematological analysis

In the subchronic toxicity test, hematological parameters were examined. The following **Table 5** shows the results of hematological examinations in male and female mice carried out at the end of the test. In hematology assessment, the 3 doses of NPL, satellite group, and saline showed no significant differences in the parameters of leucocyte count, erythrocyte count, hemoglobin, and hematocrit between groups of male mice, with *p*-value > 0.05. These results indicate that the administration of NPL for 28 days did not affect the hematological profile. The 200 mg/kgBW satellite group in this study is needed to see if any delayed toxic effects arise. In this study, the 200 mg/kgBW satellite group found no significant difference with the 200 mg/kgBW dose. This indicates that there is no delayed toxic effect that appears after stopping the administration of NPL for 14 days. The nanoparticle form of passion fruit leaf extract and the level of NPL dose usage did not affect the safety of the preparation. This is the same as the subchronic toxicity test research in a short period of time conducted by Elbesthi *et al.* [45] that increasing the dose and duration of administration does not affect hematological parameter. Research conducted by Sandhiutami *et al.* [25] on subchronic toxicity testing of curcumin nanoparticles, also obtained results that there were no significant differences in the hematological parameters of male mice and female mice.

Table 5 Haematological examination of male and female mice.

Parameters	Unit	Group					
		Normal	NPL-50	NPL-100	NPL-200	Satellite NPL-200	Reference [46]
Male							
WBC	10^3/µL	7.76 ± 0.34	7.20 ± 0.44	7.44 ± 1.05	8.18 ± 1.28	7.68 ± 0.72	3.5 - 9.7
RBC	10^6/µL	7.64 ± 0.50	7.90 ± 0.51	7.96 ± 0.37	8.0 ± 0.64	8.17 ± 0.93	6.5 - 11.5
Hb	g/dL	14.20 ± 0.51	13.68 ± 0.66	14.24 ± 0.69	14.26 ± 0.54	14.72 ± 1.58	13.6 - 16.8
HCT	%	37.96 ± 0.71	37.84 ± 0.66	38.30 ± 1.76	37.60 ± 1.55	39.28 ± 1.90	36.9 - 46.9
MCV	fL	47.86 ± 1.24	48.34 ± 0.71	47.98 ± 1.11	49.30 ± 2.27	48.26 ± 1.70	44.5 - 49.7
MCH	Pg	16.86 ± 0.64	16.88 ± 1.04	17.16 ± 0.60	16.86 ± 0.48	17.22 ± 0.57	16.1 - 18.6
MCHC	g/dL	33.84 ± 0.88	34.52 ± 0.63	33.78 ± 0.61	34.34 ± 0.61	34.30 ± 0.52	32.9 - 37.5
PLT	10^3/µL	810.6 ± 47.56	781.8 ± 71.42	838.6 ± 49.13	895.6 ± 148.57	814.6 ± 86.19	700 - 1400

Parameters	Unit	Group					
		Normal	NPL-50	NPL-100	NPL-200	Satellite NPL-200	Reference [46]
Female							
WBC	10^3/µL	7.72 ± 0.64	7.62 ± 0.55	7.46 ± 1.35	8.24 ± 1.46	8.08 ± 1.33	3.5 - 9.7
RBC	10^6/µL	8.53 ± 0.51	9.12 ± 0.32	8.99 ± 0.39	8.62 ± 0.63	8.04 ± 0.71	6.5 - 11.5
Hb	g/dL	13.94 ± 0.32	14.38 ± 0.73	14.40 ± 0.77	14.60 ± 0.72	13.96 ± 0.65	13.6 - 16.8
HCT	%	39.70 ± 0.65	39.34 ± 0.78	38.52 ± 1.06	39.72 ± 1.84	39.48 ± 1.19	36.9 - 46.9
MCV	fL	46.18 ± 0.64	46.52 ± 1,03	47.60 ± 1.39	47.24 ± 1.27	46.40 ± 1.24	44.5 - 49.7
MCH	Pg	17.48 ± 0.43	17.70 ± 0.70	17.20 ± 0.85	16.98 ± 0.61	17.34 ± 0.74	16.1 - 18.6
MCHC	g/dL	35.38 ± 1.09	35.10 ± 0.69	34.46 ± 0.71	34.60 ± 0.69	35.40 ± 0.98	32.9 - 37.5
PLT	10^3/µL	783.4±12.95	774.8 ± 75.03	798.4 ± 70.08	818±110.03	808.6±62.72	700 - 1400

Data are expressed as the mean ± SD; N = 5 mice per group. WBC: White blood cell count; RBC: Red blood cell count; Hb: Hemoglobin concentration; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: MCH concentration; PLT: Platelet count; NPL: Nanoherbal passionfruit leaves; Satellite NPL-200: A dose of 200 mg/kgBW, for observation of late toxicity signs on day 42.

Biochemical function parameters

In the subchronic toxicity test, clinical biochemical parameters (**Table 6**) were examined to determine the effect of 28 days of NPL administration on liver and kidney function. At the same time, the serum biochemical parameters ALT, AST, BUN, and Creatinine showed no significant difference ($p > 0.05$) in the normal group (saline), NPL-50, NPL-100, NPL-200, and Satellite NPL-200. Based on the data above, it is known that there was an increase in the AST and ALT values in mice, where the increase was linear with increasing NPL dose, but was still within normal limits according to the reference value. AST and ALT enzymes have the main function as biomarkers or markers of liver disorders. Differences in AST and ALT levels between groups are not indicative of liver damage but may be due to heavier liver work. If there is an increase in AST and ALT values in the blood sample, it indicates a number of lysed cells. The higher ALT and AST values mean that there is a disruption of hepatocyte cell integrity and more cells are damaged [47].

In this study, it was found that the values of Albumin, BUN and creatinine levels were in accordance with the reference values. ANOVA test showed no significant difference in the values of albumin, BUN and creatinine between groups of male mice ($p > 0.05$) and female mice ($p > 0.05$). The kidney function parameters between the normal group and the treated group, there is a tendency to increase in the treatment group. This occurs because the kidney function to eliminate the substance is getting heavier with increasing doses. These changes did not affect the function of the kidney organs because the creatinine and BUN values were still within the reference values.

Our data and the available knowledge indicate that chitosan polymer-based nanoparticles are extremely safe, non-toxic, biodegradable, and offer other significant advantages [34]. When compared to the saline treatment/normal group, all haematobiochemical values were within the normal physiological range at all doses.

Table 6 Clinical Biochemical examination of male and female mice.

Parameters	Unit	Group					Reference [46]
		Normal	NPL-50	NPL-100	NPL-200	Satellite NPL-200	
Male							
ALT	uL	33.32 ± 3.24	33.48 ± 3.51	35.54 ± 4.70	38.1 ± 6.00	36.42 ± 4.81	30 - 100
AST	uL	106.62 ± 12.07	107.12 ± 11.98	111.26 ± 11.88	115.98 ± 17.65	114.74 ± 16.19	75 - 300
Albumin	g/dL	1.72 ± 0.56	1.90 ± 0.19	2.01 ± 0.44	2.30 ± 0.31	2.04 ± 0.37	1.6 - 2.6
BUN	mg/dL	16.56 ± 2.41	18.92 ± 1.44	19.64 ± 0.86	21.64 ± 1.40	19.56 ± 0.80	10 - 30
Creatinine	mg/dL	0.37 ± 0.09	0.39 ± 0.04	0.49 ± 0.08	0.65 ± 0.27	0.57 ± 0.14	0.3 - 1
Female							
ALT	uL	34.87 ± 0.15	35.98 ± 3.95	37.18 ± 3.31	39.72 ± 3.57	38.36 ± 3.37	30 - 100
AST	uL	109.14 ± 10.51	111.08 ± 15.73	117.36 ± 18.96	124.66 ±15.23	121.56 ± 12.89	75 - 300
Albumin	g/dL	1.73 ± 0.32	1.86 ± 0.20	1.94 ± 0.18	2.17 ± 0.26	2.02 ± 0.24	1.6 - 2.6
BUN	mg/dL	16.36 ± 2.45	18.28 ± 1.55	20.86 ± 0.88	22.88 ± 1.89	19.90 ± 1.33	10 - 30
Creatinine	mg/dL	0.40 ± 0.05	0.42 ± 0.08	0.45 ± 0.05	0.51 ± 0.29	0.43 ± 0.04	0.3 - 1

Data are expressed as the mean ± SD; N = 5 mice per group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Urea nitrogen; NPL: Nanoherbal passionfruit leaves; Satellite NPL-200: a dose of 200 mg/kgBW, for observation of late toxicity signs on day 42

Histopathology

The H&E staining of the hepar and renal tissues is depicted in **Figure 4**. On the 28th day, the histopathological changes were examined. No fat degeneration occurred, no liver damage in the form of lymphocyte infiltration and no necrosis in liver cells with changes in the cytoplasm and cell nucleus pada liver in the group that received a saline solution, NPL-50, NPL-100, and NPL-200. No appreciable changes were noticed in the liver in all groups. Similar to the kidneys, the proximal tubule, which is defined by the loss of the brush border - that is, the microvilli - and the nucleus, does not exhibit cell necrosis. Assuming that the chitosan utilized as a polymer in the production of nanoherbal NPL is a naturally occurring polysaccharide, it is harmless and has no harmful effects on the liver's metabolic processes or the kidneys' expulsion of waste products.

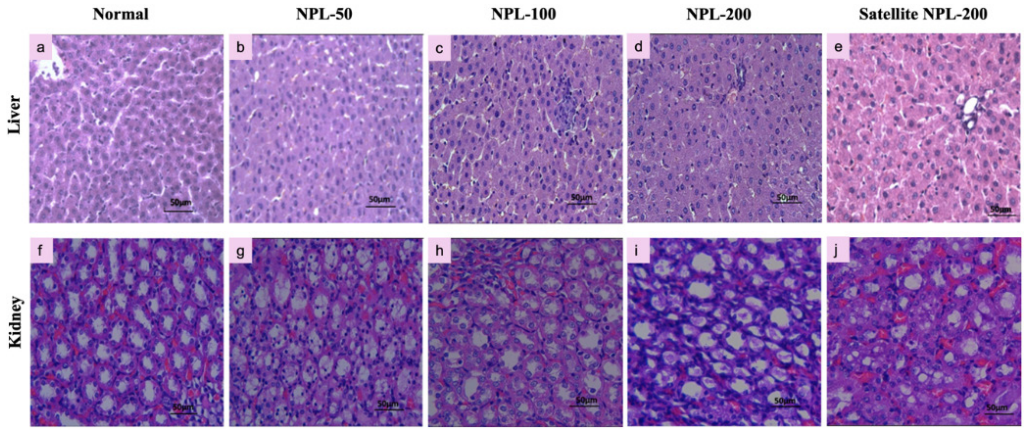

Figure 4 Effect of repeated 28-day administration of Nanoherbal passionfruit leaves on the histopat of tissues in mice. (a) - (e) Liver; (f) - (j) Kidney: Normal; Nanoherbal passionfruit leaves at doses 50, 100, 200, and satellite 200 mg/kgBW (Haematoxylin and Eosin staining; 400×.

Assay for analgesic effect NPL in vivo

After testing, NPL showed a substantial change in the writhing test, according to One-way ANOVA (**Figure 5**). A post hoc analysis revealed that at all 3 NPL dosages, there is an inhibition of the quantity of abdominal writhing ($p < 0.05$). The highest level of inhibition was seen at NPL-200. The results were similar to those of the prescription medication diclofenac sod., which similarly significantly reduced the writhing in the abdomen. The average number of writhes in mice every 5 min from each group can be seen in **Figure 5**. It can be seen that the number of writhing decreased at the 30th min. This shows that NPL can inhibit the increase in the number of writhing in mice. Assessment of the effectiveness of analgesic drugs in addition to seeing the increase and decrease in the number of writhing in mice, can also be seen from the AUC (Area Under Curve) value (**Table 7**). The smaller the AUC value, the greater the effectiveness of an analgesic drug. In this study, it can be seen that all doses and positive controls have analgesic effects because they have lower AUC values than negative controls. The 200 mg/KgBW dose has the lowest AUC compared to the other dose groups, indicating that the 200 mg/KgBW dose has better effectiveness as an analgesic.

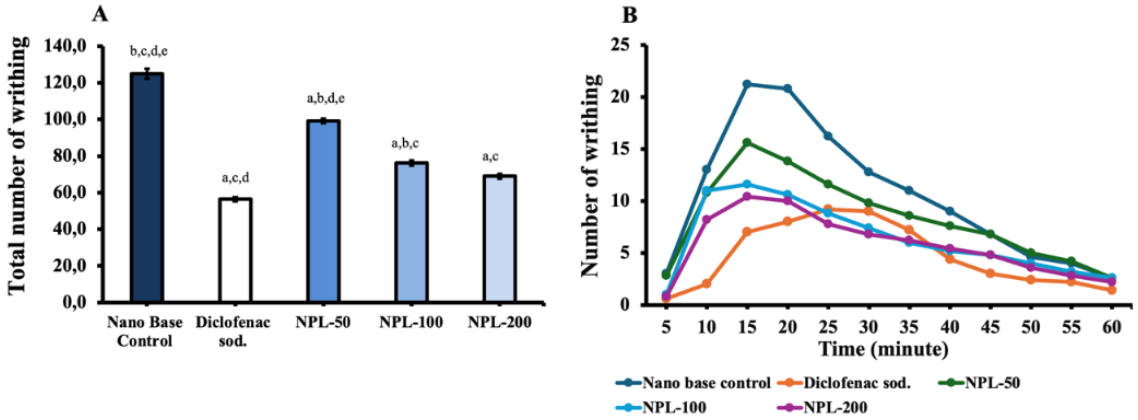

Figure 5 (A) Total number of writhing (a) p-value < 0.05 vs. Nano base control; (b) p-value < 0.05 vs. diclofenac sod.; (c) p-value < 0.05 vs. NPL-50; (d) p-value < 0.05 vs. NPL-100; (d) p-value < 0.05 vs. NPL-200; (B) Graph of the relationship between time and average number of writhes. NPL: Nanoherbal passionfruit leaves.

Table 7 Analgesic Activity and effectiveness analgesic of nanoherbal passionfruit leaves

Group	AUC (Mean ± SD)	Analgesic activity (%)	Effectiveness analgesic (%)
Nano Base Control	610.9 ± 91.51	-	-
Diclofenac sod.	277.0 ± 30.69	54.66	-
NPL-50	482.5 ± 42.72	21.02	38.46
NPL-100	380.0 ± 31.26	37.80	69.15
NPL-200	327.5 ± 36.83	47.39	84.87

Data are expressed as the mean ± SD; N = 5 mice per group. AUC: Area under curve; NPL: Nanoherbal passionfruit leaves

The test results in **Table 7** can be seen that the positive control has better inhibition of the number of writhes compared to the test preparation group. Based on **Table 7**, it can also be seen that NPL-200 has the greatest inhibition of the number of writhes compared to other doses. If the percentage of NPL effectiveness is calculated when compared to the standard drug diclofenac sod, it is found that the NPL-200 group has better effectiveness as an analgesic (84.87 %) compared to NPL-50 (37.80 %) and NPL-100 (69.15 %). This current study the safety and analgesic potential of NPL in animal models. The results of the analgesic activity suggest that NPL exhibited significant inhibition of pain response in acetic acid-induced pain models. NPL had an impact similar to that of diclofenac sod. in the reduction of rats' acetic acid-induced pain, indicating the function of NPL in blocking the cyclooxygenase or lipoxygenase route - the general pathway used by most used peripherally acting analgesic medications [48]. This happens because passionfruit leaves contain flavonoids. Flavonoid compounds can inhibit the cyclooxygenase enzyme resulting in reduced production of prostaglandins by arachidonic acid to reduce the onset of pain [49].

In previous studies, passionfruit leaf extract showed the ability to reduce pain and have anti-inflammatory effects [5]. The preparation of *P. edulis* nanoherbal is expected to have better activity. The form of nanoparticles has enormous promise in medical applications. Because of their high carrier capacity, ability to bind both hydrophilic and hydrophobic substances with ease, variability in size and shape, and ability to form stable interactions with ligands, nanoparticles are useful platforms for the targeted and controlled delivery of micro- and macromolecules in disease therapy [13]. However, further studies are needed to investigate the pharmacokinetics (absorption, distribution, metabolism and excretion) NPL, as well as the active compound uptake in target organs. The mechanism of action of NPL on target organs against the inhibition of cytokines such as arachidonic acid, prostaglandins, interleukins etc., should also be investigated. Since we use crude extracts that can have batch variations of bioactive components, depending on the farming area, harvesting time, and season will affect the standard of raw materials and nanoparticle characterization so we need a standardized source of raw materials. NLP in the form of nanosuspension cannot be used practically by the public so the best pharmaceutical dosage form needs to be developed.

Conclusions

Nanoherbal *Passiflora edulis* leaves have been developed successfully with the ionic gelation method. Conclusively, we reported a nanoparticles formulation composed of chitosan-sodium tripolyphosphate that was able to achieve a particle size of 187.6 nm, PI of 0.626, potential zeta of +31.2 mV, spherical shape in TEM images with Entrapment Efficiency of 50.96 %. In vivo analgesic studies for oral administration NPL-200 has an analgesic activity of 47.39 % compared to the negative control and has an analgesic effectiveness of up to 84.87 % compared to diclofenac sod. Overall, the data indicated that NPL is a powerful and safe medication analgesic.

Acknowledgments

We are grateful to The Ministry of Education, Culture, Research, and Technology, The Republic of Indonesia provided support for this work under the Fundamental Regular Research 2024 program with number 0034/LPPM/UP/VI/2024.

References

[1] JK Kim, KH Kim, YC Shin, BH Jang and SG Ko. Utilization of traditional medicine in primary health care in low-and middle-income countries: a systematic review. *Health Policy Plan.* 2020; **35**, 1070-83.

[2] KMM Koriem. Importance of herbapassiflorae in medicinal applications: Review on experimental and clinical pharmacology. *Biointerface Res. Appl. Chem.* 2021; **11**, 12886-900.

[3] X He, F Luan, Y Yang, Z Wang, Z Zhao, J Fang, M Wang, M Zuo and Y Li. Passiflora edulis: an insight into current researches on phytochemistry and pharmacology. *Front. Pharmacol.* 2020; **11**, 617.

[4] S Rai, JC Nagar and M Mukim. Pharmacological and medicinal importance of passiflora edulis: A review. *Int. J. Res. Rev.* 2022; **9**, 341-9.

[5] NMD Sandhiutami, Y Desmiaty, MH Fakhran and Fahleni. IAI special edition: antinociceptive and anti-inflammatory effect of passiflora edulis leaves extract in acetic acid-induced pain and carrageenan-induced paw oedema in vivo. *Pharm. Educ.* 2024; **24**, 78-85.

[6] H Yuan, Q Ma, L Ye and G Piao. The traditional medicine and modern medicine from natural products. *Molecules* 2016; **21**, 559.

[7] JB Calixto. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Braz. J. Med. Biol. Res.* 2000; **33**, 179-89.

[8] X Cai, Z Fang, J Dou, A Yu and G Zhai. Bioavailability of quercetin: problems and promises. *Curr. Med. Chem.* 2013; **20**, 2572-82.

[9] J Zhao, J Yang and Y Xie. Improvement strategies for the oral bioavailability of poorly water-soluble flavonoids: An overview. *Int. J. Pharm.* 2019; **570**, 118642.

[10] AR Bilia, V Piazzini, C Guccione, L Risaliti, M Asprea, G Capecchi and MC Bergonzi. Improving on nature: the role of nanomedicine in the development of clinical natural drugs. *Planta Med.* 2017; **83**, 366-81.

[11] MF Manzoor, A Hussain, A Sameen, A Sahar, S Khan, R Siddique, RM Aadil and B Xu. Novel extraction, rapid assessment and bioavailability improvement of quercetin: A review. *Ultrason. Sonochem.* 2021; **78**, 105686.

[12] R Shegokar. *Chapter 2 - Role of nanocarriers and their surface modification in targeting delivery of bioactive compounds*. *In*: H Rachmawati, A Larasati, AC Adi and R Shegokar (Eds). Nanopharmaceuticals. Elsevier Science, Amsterdam, Netherland, 2020.

[13] AA Yetisgin, S Cetinel, M Zuvin, A Kosar and O Kutlu. Therapeutic nanoparticles and their targeted delivery applications. *Molecules* 2020; **25**, 2193.

[14] R Kumar and M Sharma. Herbal nanomedicine interactions to enhance pharmacokinetics, pharmacodynamics, and therapeutic index for better bioavailability and biocompatibility of herbal formulations. *J. Mater. Nanosci.* 2018; **5**, 35-60.

[15] J Zhang, K Hu, L Di, P Wang, Z Liu, J Zhang, P Yue, W Song, J Zhang, T Chen, Z Wang, Y Zhang, X Wang, C Zhan, Y Cheng, X li, Q Li, J Fan, Y Shen, J Han and H Qiao. Traditional herbal medicine and nanomedicine: Converging disciplines to improve therapeutic efficacy and human health. *Adv. Drug. Deliv. Rev.* 2021; **178**, 113964.

[16] SR Alizadeh, N Savadkouhi and MA Ebrahimzadeh. Drug design strategies that aim to improve the low solubility and poor bioavailability conundrum in quercetin derivatives. *Expert Opin. Drug Discov.* 2023; **18**, 1117-32.

[17] F Araujo, N Shrestha, PL Granja, J Hirvonen, HA Santos and B Sarmento. Safety and toxicity concerns of orally delivered nanoparticles as drug carriers. *Expert Opin. drug Metab. Toxicol.* 2015;

11, 381-93.

[18] C Ceylan, ME Tatlipinar, S Tuccar, GZ Omurtag and FJ Akbuga. *Evaluation of the in vivo Preclinical Toxicity of Targeted Nanoparticles*. 1st ed. Jenny Stanford Publishing, Dubai, United Arab Emirates, 2021.

[19] C Egbuna, VK Parmar, J Jeevanandam, SM Ezzat, KC Patrick-Iwuanyanwu, CO Adetunji, J Khan, EN Onyeike, CZ Uche, M Akram, MS Ibrahim, NME Mahdy, CG Awuchi, K Saravanan, H Tijjani, UE Odoh, M Messaoudi, JC Ifemeje, MC Olisah, NJ Ezeofor, CJ Chikwendu and CG Ibeabuchi. Toxicity of nanoparticles in biomedical application: Nanotoxicology. *J. Toxicol.* 2021; **2021**, 9954443.

[20] R Abbasi, G Shineh, M Mobaraki, S Doughty and L Tayebi. Structural parameters of nanoparticles affecting their toxicity for biomedical applications: A review. *J. Nanopart. Res.* 2023; **25**, 43.

[21] T Jansen, L Claassen, IV Kamp and DRM Timmermans. All chemical substances are harmful: public appraisal of uncertain risks of food additives and contaminants. *Food Chem. Toxicol.* 2020; **136**, 110959.

[22] J Boutrand. *Biocompatibility and Performance of Medical Devices*. *In*: WH De Jong, JW Carraway and RE Geertsma (Eds). *In vivo* and *in vitro* testing for the biological safety evaluation of biomaterials and medical devices. Woodhead Publishing, Cambridge, 2020.

[23] A Gissi, K Louekari, L Hoffstadt, N Bornatowicz and AM Aparicio. Alternative acute oral toxicity assessment under REACH based on sub-acute toxicity values. *ALTEX-Alternatives Anim. Exp.* 2017; **34**, 353-61.

[24] BPBP Obat and M Nomor. *Tahun 2022 on Guidelines for preclinical toxicity tests in vivo*. Indonesian Food and Drug Authority, Indonesia, 2022, p. 1-53.

[25] NMD Sandhiutami, RS Dewi, S Khairani and SAM Widyadari. Safety evaluation of the development of a curcumin nanoparticle formula in mice and *in-vitro* antioxidant potential safety evaluation of the development of a curcumin nanoparticle formula in mice and *in-vitro* antioxidant potential. *Jurnal Ilmu Kefarmasian Indonesia.* 2022; **20**, 63-72.

[26] MCLD Carmo, IM Martins, AER Magalhaes, MRM Junior and JA Macedo. Passion fruit (Passiflora edulis) leaf aqueous extract ameliorates intestinal epithelial barrier dysfunction and reverts inflammatory parameters in Caco-2 cells monolayer. *Food Res. Int.* 2020; **133**, 109162.

[27] MR Priamsari and CH Kristanti. Effect of Solvent Type on the yield amount and total flavonoid content of yellow passion fruit peel extract (Passiflora edulis var. flavicarpa). *Indones. J. Med. Sci.* 2023; **10**, 37-42.

[28] W Arozal, M Louisa, D Rahmat, P Chendrana and NMD Sandhiutami. Development, characterization and pharmacokinetic profile of chitosan-sodium tripolyphosphate nanoparticles based drug delivery systems for curcumin. *Adv. Pharm. Bull.* 2021; **11**, 77-85.

[29] MH Zarei, Z Lorigooini, HA Khoei and E Bijad. Acute oral toxicity assessment of galbanic acid in albino rat according to OECD 425 TG. *Toxicol. Rep.* 2023; **11**, 111-5.

[30] NMD Sandhiutami, S Khairani, RS Dewi, ZR Hakim and AR Pradani. Anti-inflammatory and analgesic activity of musa balbisiana peels *in vivo*. *Borneo J. Pharm.* 2022; **5**, 81-92.

[31] NMD Sandhiutami, AT Atayoglu, Y Sumiyati, Y Desmiaty and RA Hidayat. The combination of colocasia esculenta L. and zingiber officinale potentially inhibits inflammation and pain. *Jurnal Ilmu Kefarmasian Indonesia* 2023; **21**, 81-9.

[32] AVV Nikezic, AM Bondzic and VM Vasic. Drug delivery systems based on nanoparticles and related nanostructures. *Eur. J. Pharm. Sci.* 2020; **151**, 105412.

[33] R Kumar, SV Dalvi and PF Siril. Nanoparticle-based drugs and formulations: Current status and

emerging applications. *ACS Appl. Nano Mater.* 2020; **3**, 4944-61.

[34] CP Jimenez-Gomez and JA Cecilia. Chitosan: A natural biopolymer with a wide and varied range of applications. *Molecules* 2020; **25**, 3981.

[35] AM Abdelgawad and SM Hudson. Chitosan nanoparticles: polyphosphates cross-linking and protein delivery properties. *Int. J. Biol. Macromol.* 2019; **136**, 133-42.

[36] S Kumar, JP Singh, D Giri and S Mishra. Effect of polydispersity on the dynamics of active brownian particles. *Phys. Rev. E* 2021; **104**, 024601.

[37] J Xu, M Yildiztekin, D Han, C Keskin, A Baran, MF Baran, A Eftekhari, CA Ava, SI Kandemir, DB Cebe, B Dag, A Beilerli and R Khalilov. Biosynthesis, characterization, and investigation of antimicrobial and cytotoxic activities of silver nanoparticles using solanum tuberosum peel aqueous extract. *Heliyon* 2023; **9**, e19061.

[38] J Zhang, Q Xiang, L Shen, J Ling, C Zhou, J Hu and L Chen. Surface charge-dependent bioaccumulation dynamics of silver nanoparticles in freshwater algae. *Chemosphere* 2020; **247**, 125936.

[39] A Yusuf, ARZ Almotairy, H Henidi, OY Alshehri and MS Aldughaim. Nanoparticles as drug delivery systems: a review of the implication of nanoparticles' physicochemical properties on responses in biological systems. *Polymers* 2023; **15**, 1596.

[40] M Soltanzadeh, SH Peighambardoust, B Ghanbarzadeh, M Mohammadi and JM Lorenzo. Chitosan nanoparticles as a promising nanomaterial for encapsulation of pomegranate (*Punica granatum* L.) peel extract as a natural source of antioxidants. *Nanomaterials* 2021; **11**, 1439.

[41] K Jafernik, A Ladniak, E Blicharska, K Czarnek, H Ekiert, AE Wiacek and A Szopa. Chitosan-based nanoparticles as effective drug delivery systems - a review. *Molecules* 2023; **28**, 1963.

[42] PN Azizah and Y Herdiana. Review article: Chitosan nanoparticles to improve nutraceutical quality. *Farmaka* 2023; **21**, 399-409.

[43] H Sadaquat, M Akhtar, M Nazir, R Ahmad, Z Alvi and N Akhtar. Biodegradable and biocompatible polymeric nanoparticles for enhanced solubility and safe oral delivery of docetaxel: *In vivo* toxicity evaluation. *Int. J. Pharm.* 2021; **598**, 120363.

[44] N Munot, U Kandekar, PS Giram, K Khot, A Patil and S Cavalu. A comparative study of quercetin-loaded nanocochleates and liposomes: formulation, characterization, assessment of degradation and in vitro anticancer potential. *Pharmaceutics* 2022; **14**, 1601.

[45] RTA Elbesthi, KY Ozdemir, Y Tacstan, S Bilen and AY Sonmez. Effects of ribwort plantain (*plantago lanceolata*) extract on blood parameters, immune response, antioxidant enzyme activities, and growth performance in rainbow trout (*oncorhynchus mykiss*). *Fish Physiol. Biochem.* 2020; **46**, 1295-307.

[46] G Silva-Santana, JC Bax, DCS Fernandes, DTL Bacellar, C Hooper, AASO Dias, CB Silva, AMD Souza, S Ramos, RA Santos,TR Pinto, MA Ramao and AL Mattos-Guaraldi. Clinical hematological and biochemical parameters in swiss, BALB/c, C57BL/6 and B6D2F1 mus musculus. *Anim. Models Exp. Med.* 2020; **3**, 304-15.

[47] NC Azubike, PU Achukwu, CN Okwuosa, DC Nwachukwu, OS Onwukwe and AO Onyemelukwe. Subacute toxicity profile of the leaves of colocasia esculenta [L. Schott] in albino rats. *Res. J. Med. Plant* 2016; **10**, 340-8.

[48] ME Jemli, SM Ezzat, M Kharbach, ES Mostafa, RA Radwan, YE Jemli, O El-Guourrami, S Ahid, Y Cherrh, A Zayed and K Alaoui. Bioassay-guided isolation of anti-inflammatory and antinociceptive metabolites among 3 Moroccan Juniperus leaves extract supported with in vitro enzyme inhibitory assays. *J. Ethnopharmacol.* 2024; **331**, 118285.

[49] N Sa'adah, N Chasanah, SDI Pertami, PD Rohmaniar, AA Adriansyah and AM Ulah. Analgesic effect of trembesi leaf extract (*Samanea saman* (Jacq.) Merr.) on white mice (*Mus musculus*). *Padjadjaran J. Dent. Res. Students* 2022; **6**, 120-6.