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Original Article

PREPARATION, CHARACTERIZATION, AND TOXICITY STUDY OF *ANDROGRAPHIS PANICULATA* ETHANOL EXTRACT POLY-LACTIC-CO-GLYCOLIC ACID (PLGA) NANOPARTICLES IN RAW 264.7 CELLS

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ABSTRACT

Objective: This research aims to prepare and characterize *Andrographis paniculata* ethanol extract nanoparticles using Poly-Lactic-co-Glycolic Acid (PLGA) and test the toxicity of the nanoparticles *in vitro* in Raw 264.7 macrophage cells.

Methods: *A. paniculata* ethanol extract-PLGA nanoparticles were prepared using the solvent evaporation method. The nanoparticles were characterized for their particle size and particle size distribution using a Particle Size Analyzer (PSA) and their zeta potential was measured using a zetasizer. The morphology of the nanoparticles was observed using a Scanning Electron Microscope (SEM). To confirm whether the ethanol extract of *A. paniculata* was loaded in the PLGA nanoparticles, it was determined using Fourier Transform Infra-Red (FTIR) and Raman spectroscopy. *In vitro* toxicity test of *A. paniculata* ethanol extract nanoparticles in Raw cells macrophage 264.7 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Results: After measurements using PSA, *A. paniculata* ethanol extract nanoparticles had an average size of 466.4 ± 31.6 nm, a Polydispersity Index (PdI) of 0.365 ± 0.03, and a zeta potential of-2.42 ± 0.91 mV. The results of observations using FTIR on *A. paniculata* ethanol extract nanoparticles show peaks at wavenumbers 712 cm^{-1}, 749 cm^{-1}, 865 cm^{-1}, 955 cm^{-1}, 1093 cm^{-1}, 2949 cm^{-1}, 1757 cm^{-1}, and 3390 cm^{-1}. This proves that *A. paniculata* ethanol extract is loaded into the nanoparticles. The results of *in vitro* toxicity tests using Raw macrophage 264.7 cells showed that the ethanol extract of *A. paniculata*-PLGA nanoparticles was not toxic.

Conclusion: PLGA-based *A. paniculata* ethanol extract nanoparticles have good characteristics as a nanotechnology-based preparation and are non-toxic when tested *in vitro* in Raw macrophage cells 264.7.

Keywords: *A. paniculata*, Ethanol extract, Nanoparticles, PLGA, Raw cells 264.7

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INTRODUCTION

Sambiloto (*Andrographis paniculata*) is, commonly known as 'King of Bitter', is a small, branching annual plant belonging to the Acanthaceae family. This plant grows widely in Southeast Asia, especially in Indonesia, India, Sri lanka, Pakistan, and Malaysia [1]. *A. paniculata* part of the medicinal plants that is the focus of development and research by institutions in Indonesia such as National Agency of Drug and Food Control (Badan Pengawas Obat dan Makanan/BPOM), Ministry of Agriculture, Association of Herbal Medicine Entrepreneurs, National Research Council, and Herbal Scientification. Apart from that, *A. paniculata* is also one of the natural commodities in high demand worldwide [2]. Furthermore, *A. paniculata* is frequently consumed due to the presence of active chemical compounds such as lactones and flavonoids [3]. These compounds can act as antioxidants. Cell damage caused by the presence of free radicals can be overcome by antioxidants. Flavonoids function as natural antioxidants that can capture free radicals such as Reactive Oxygen Species (ROS) or Reactive Nitrogen Species (RNS). It can repair the condition of damaged tissue, regenerate cells to a normal state or towards healing from disease [4]. The main active compound contained in *A. paniculata* is andrographolide, which is known to have many pharmacological activities, including anti-inflammatory, anti-cancer, anti-obesity, anti-diabetes, and other activities [5]. Moreover, *A. paniculata,* also known as has hepatoprotective activity [6].

Due to the large number of pharmacological activities in *A. paniculata*, this plant has a great potential as a treatment agent for many diseases as an alternative to synthetic chemical drugs [7]. One approach that can be taken to increase the potential of *A. paniculata* is through nanotechnology. Via a nanotechnology approach, drugs can improve their solubility, permeation ability, penetration ability, etc. to increase the therapeutic efficacy and minimize the adverse effects of the drug [8].

Nanotechnology approaches to medicine exist in several variations, one of which is polymeric nanoparticles. A polymer of interest that can be used in nanomedicine applications is Poly-Lactic-co-Glycolic Acid (PLGA). PLGA was chosen because it is biodegradable and biocompatible, and it has been clinically approved by the Food and Drug Administration (FDA) [9]. PLGA has also been widely used as a nanoparticle base in drug delivery systems in various biomedical applications such as inflammation therapy, vaccination, cancer, and other diseases [10]. These excellent properties of PLGA are expected able to increase the pharmacological potential of *A. paniculata* as an alternative therapy for various types of chronic diseases [11].

Therefore, in this study, *A. paniculata* ethanol extract was synthesized into nanoparticles using PLGA. then, subsequently, the samples were characterized using various physical and chemical methods. The toxicity *in vitro* test was performed using an MTT assay on Raw 264.7 cells.

MATERIALS AND METHODS

Instruments and materials

The UV-Vis spectrophotometer (Drawell), pH meter, particle size

analyzer (PSA, Malvern Instruments ltd.), Fourier Transform Infra-Red (FTIR, Thermo Scientific Nicolet iS10), Raman spectroscopy, and Scanning Electron Microscopy (SEM, SEM-EDX Phenom Desktop ProXL) were the instruments used in this study.

The materials used in this research are sambiloto *(A. paniculata)* herbs purchased from PT Global Sarana Saintifik Indonesia which had been confirmed by the laboratory of Plant Taxonomy Faculty of Biology Universitas Jenderal Soedirman as certificate number T/433/UN23.6.10/TA.00.01/2021, andrographolide standard (Markherb, Indonesia], PLGA was purchased from Merck, aqua deionized [Onelab, Indonesia], Polyvinyl Alcohol (PVA), KCl, KBr, ethyl acetate [Merck], Raw 264.7 macrophages cell, Dulbecco's Modified Eagle Medium High Glucose (DMEM-HG) [Gibco], Fetal Bovine Serum (FBS) [Merck], penicillin-streptomycin antibiotics, lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and sodium dodecyl sulfate (SDS) [Merck].

Methods

Extraction of *A. paniculata* herbs

Sambiloto (*A. paniculata*) herbs were ground into powder using a blender. The powder is mixed with ethanol 70% in a ratio of 1:7. 1 kg of *A. paniculata* powder was weighed and mixed with 7 l of ethanol 70%. The herbs powder was extracted by maceration for 3 x 24 hours, with periodic stirring at room temperature. The filtrate formed was filtered, then evaporated in a water bath to be a concentrate, solvent-free extract was obtained.

Confirmation of andrographolide in *A. paniculata* ethanol extract

A motion phase consisting of n-hexane: ethyl acetate (2:8) was prepared. After that, the motion phase was placed into a chamber and left until saturated. The sample solution was manufactured by dissolving 50 mg of *A. paniculata* extract in methanol up to 50 ml. The standard solution was manufactured by dissolving 5 mg andrographolide standard in 5 ml ethanol 70%. On the Thin layer Chromatography (TLC) plate (silica gel 60 F_{254}), the sample solution and standard solution were spotted side by side, then placed into the chamber, eluted until the boundary mark was taken out, and left to dry. Following to observed under UV light at 254 nm [12].

Preparation of *A. paniculata* ethanol extract nanoparticles using PLGA

The ethanol extract of *A. paniculata* as the active compound was weighed amount of 2 g and then dissolved in 100 ml of deionized water. PVA powder was weighed in a ratio of 1:100 (PVA (g): aqua deionized (ml)) to prepare 5% PVA solution as a liquid phase and 0.3% PVA solution to harden the PLGA nanoparticles.

To obtain the optimal formula, we vary the PLGA concentration. The PLGA weighed 2 mg, 4 mg, and 6 mg. Each of the PLGA was mixed with 100 µl of ethyl acetate in an Eppendorf tube. After that, 100 µL of *A. paniculata* extract was mixed in the PLGA-ethyl acetate mixture by dripping, continuing to agitate with vortex for 2 minutes. This part is called the organic phase.

Prepare the liquid phase in an Eppendorf tube. The organic phase is dropped gently into the liquid phase in the Eppendorf tube, then continues to agitate with vortex for 2 minutes. The formulation of *A. paniculata* ethanol extract nanoparticles using PLGA as a polymer can be seen in table 1.

To harden the nanoparticles was resulted, prepare PVA 0.3% solution in a Beaker glass completed with the magnetic stirrer (±15 ml). The mixture of the liquid phase and organic phase was dropped gently into the PVA 0.3% solution while continuing the stirring. Stir overnight until the smell of ethyl acetate disappears. For the final step, centrifuge the mixture out for 10 minutes at 10,000 rpm. The resulting precipitate was dissolved with 1 ml of aqua deionized in an Eppendorf tube [11].

Table 1: Formulation of *A. paniculata* ethanol extract using PLGA

Materials	Formula 1	Formula 2	Formula 3
PLGA	2 mg	4 mg	6 mg
PVA 5%	100 ml	100 ml	100 ml
PVA 0.3%	±15 ml	±15 ml	±15 ml
Ethyl acetate	100 µl	100 µl	100 µl
A. paniculata ethanol extract	100 µl	100 µl	100 µl

Characterization of *A. paniculata* ethanol extract nanopaticles

The sample characterization of *A. paniculata* nanoparticles included morphology, particle size and distribution of particle size, zeta potential, FTIR, and Raman spectroscopy.

The particle size and particle size distribution of the nanoparticles were determined using a particle size analyzer (PSA). A sample of 50 µL was taken and then diluted with 1 ml of distilled water. The sample is dropped into a cuvette; then the cuvette is put into the PSA. The results of these measurements can be seen in a computer program connected to the PSA [13].

The zeta potential value was determined using the Malvern Zetasizer. The amount of 50 µl of the sample was taken and then diluted with 1 ml of Potassium Chloride (KCl)10 mmol solution. The sample is dripped into a cuvette; then, the cuvette is put into a zetasizer. The tool is automatically connected to a computer and will measure the zeta potential value of the nanoparticles [11].

Morphological characterization of *A. paniculata* nanoparticles was observed using a Scanning Electron Microscope (SEM). Nanoparticle samples were dried using freeze dryer, then the powder sample was placed in the holder and analyzed [11].

Fourier Transform Infra-Red (FTIR) analysis

The presence or absence of the active substance in *A. paniculata* can be seen using a Fourier Transform Infra-Red (FTIR). The nanoparticle samples were dried to be powder using freeze dryer. Then the sample was mixed with Potassium Bromide (KBr) and formed into pellets. Afterward, the sample was analyzed using FTIR in the wavelength range 4000-400 cm^{-1} [14, 15].

Raman spectroscopy

Nanoparticles samples are freeze-dried then mixed with KBr to form pellets. The freeze-dried *A. paniculata* ethanol extract powder was determined in Raman spectroscopy to obtain the data of active compound-PLGA grafting [16].

Cell viability test (MTT assay)

RAW 264.7 macrophage cells were seeded in 96-well plates amount of 10,000 cells/200µl in each well complete DMEM-HG media and incubated overnight. Samples were added into each well with different concentrations in 3 replications and then incubated overnight. After that, the medium in each well was discarded and replaced with 200 µl of MTT solution in the medium (0.5 mg/ml) to form violet-blue formazan crystals. After incubation for 4 h, 50 µl of SDS 20% solution was added into each well and incubated using a shaker incubator at 37 °C overnight to dissolve the formazan crystals formed. The absorbance of the formazan crystals formed was measured using a microplate reader at a wavelength of 570 nm [17].

Data analyses

The results, including particle morphology, particle size, distribution of particle size, zeta potential, FTIR peak, and Raman Spectroscopy, was

analyzed by descriptive quantitative. One-way ANOVA was used to analyze the cell viability at a 95% confidence level. The Tukey HSD test is performed if the results show a significant difference in the data.

RESULTS AND DISCUSSION

Confirmation of andrographolide in *A. paniculata* ethanol extract

The spot is emerged for the ethanol extract of *A. paniculata*, which is closely resemble to the andrographolide spot as a marker compound. A fair comparison of these two spots can be seen in fig. 1.

From the spots obtained, the Retention factor (Rf) value was then calculated. The Rf value for the ethanol extract of *A. paniculata* was 0.43±0.06 and the Rf value for andrographolide was 0.42±0.03. These Rf values further strengthen the evidence that the ethanol extract of *A. paniculata* contains andrographolide. The Rf value in the chromatogram of this study meets the requirements for a good Rf value, namely between 0.2-0.8 [18]. Andrographolide is known as the main chemical compound of *A. paniculata* which is known to have many pharmacological activities [3].

Characteristics of *A. paniculata* ethanol extract nanoparticles

Based on the PSA measurement results, the particle size, particle size distribution, and zeta potential of *A. paniculata* ethanol extract nanoparticles were obtained as shown in table 2.

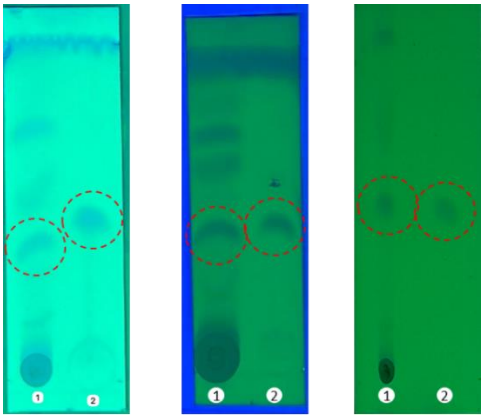

Fig. 1: Images of *A. paniculata* ethanol extract spot (1) and andrographolide spot (2)

Table 2: The particle size, particle size distribution, and zeta potential of *A. paniculata* ethanol extract nanoparticles

Sample	Z-Average (d. nm)	PdI	ZP (mV)
Nanosambiloto	466.4±31.6	0.365±0.03	-2.42±0.91
Empty-NPs	441.7±21.41	0.25±0.01	-1.03±0.38

Data are given as mean±SD, n=3.

The results of these measurements show that the ethanol extract nanoparticles of *A. paniculata* are proven to have good nanoparticle properties. The average particle size of 466±31.6 nm is categorized in the nanoparticle range even though it is not in line with the common of the polymeric nanoparticles size range is about 200 nm [19]. The zeta potential of *A. paniculata* ethanol extract nanoparticles is classified as minus low; perhaps for further development the value can be increased is supposed more stable nanoparticles physically can be obtained.

Morphological observations of *A. paniculata* extract nanoparticles using SEM can be seen in fig. 2.

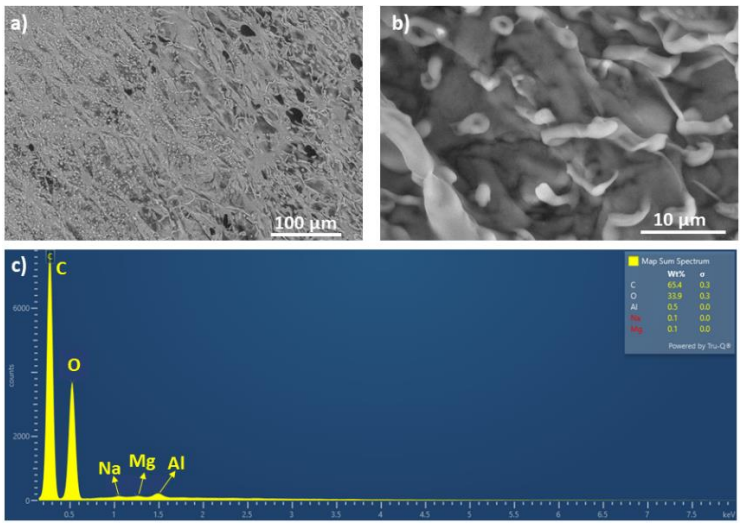

Fig. 2: SEM images of PLGA-*A. paniculata* ethanol extract nanoparticles a) 250 times magnification, b) 3000 times magnification and c) EDS spectrum

The morphology of PLGA-*A. paniculata* ethanol extract nanoparticles were examined using SEM as shown in fig. 1. The SEM image with 250 times magnification (fig. 1a) exhibits randomized nanoparticles decorated onto the branches-like samples. Furthermore, the SEM image under 3000 times magnification displays the presence of PLGA-*A. paniculata* ethanol extract nanoparticles in irregular shape. We postulate this observation is mainly contributed from the PLGA, generating rough surfaces [20]. EDS spectrum is utilized to estimate the elemental composition of PLGA-*A. paniculata* ethanol extract nanoparticles outlined in fig. 1c. Here we note that the sample is dominated by the contribution of carbon and oxygen with the fraction of 65.4 wt% and 33.9 wt%, respectively.

Confirmation of *A. paniculata* ethanol extract loaded into PLGA nanoparticles

To confirm that the ethanol extract of *A. paniculata* was loaded in the PLGA nanoparticles, we used FTIR analysis and Raman spectroscopy. The results of FTIR analysis of *A. paniculata* ethanol extract nanoparticles can be seen in fig. 3.

The summary of the functional group comparison between PLGA alone, ethanol extract of *A. paniculata*, and ethanol extract of *A. paniculata*-PLGA nanoparticles [21, 22] is displayed in table 3.

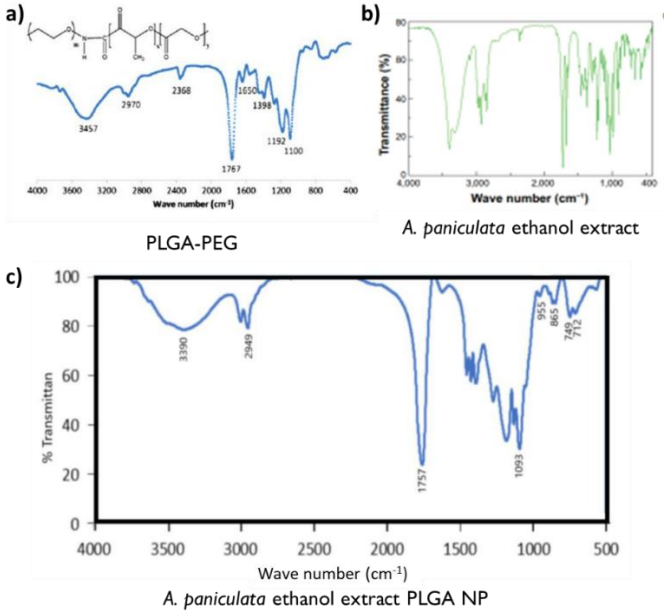

Fig. 3: The results of FTIR spectrum analysis: a) PLGA-PEG (polyethylene glycol) spectrum; b) *A. paniculata* ethanol extract spectrum; c) Nanoparticles of *A. paniculata* ethanol extract-PLGA spectrum

Table 3: The comparison of PLGA spectrum, *A. paniculata* ethanol extract spectrum, and *A. paniculata* ethanol extract nanoparticles

Bonds and absorption area (cm^{-1})	Empty PLGA nanoparticles	Ethanol extract of *A. paniculata*	*A. paniculata* ethanol extract-PLGA nanopaticles
C-H (alkene, aromatic) (675-995) strong-medium	681; 761; 832; 918	871	712; 749; 865; 955
C-O (alcohol, carboxylic acid, ester, ether) (1000-1300) strong	1082; 1130	1080	1093
C-H (alkane) (2850-2960) strong-medium	2916; 2956	2918	2949
O-H (alcohol, phenol, carboxylic acid) (3200-3600) medium	3462	3361	3390
C=O (aldehyde, ketones) (1640-1760) strong	1754	1649	1757
C=C (alkene) (1610-1680) medium-weak	-	1649	-
C=C (aromatic) (1500-1600) medium-weak	-	1544	-

Table 3 shows the band spectrum between PLGA-*A. paniculata* ethanol extract nanoparticles, empty PLGA nanoparticles, and *A. paniculata* ethanol extract show a peak shift that is not too wide. This indicates that there are no major drug-polymer interactions that could alter the efficacy of any functional group [23].

The results of the spectrum of PLGA-*A. paniculata* nanoparticles show the peaks of *A. paniculata* are at peaks 712 cm^{-1}, 749 cm^{-1}, 865 cm^{-1}, 955 cm^{-1}, 1093 cm^{-1}, 2949 cm^{-1}, 1757 cm^{-1}, and 3390 cm^{-1}. This proves that *A. paniculata* ethanol extract is contained in the PLGA nanoparticles [23].

We also verified the loading of *A. paniculata* ethanol extract into nanoparticles using Raman spectroscopy analysis, the results of which can be seen in fig. 4.

Referring to fig. 4, the ethanol extract of *A. paniculata* is loaded into the PLGA nanoparticles. Distinctive Raman frequencies or shifts reveal insights into the chemical composition of a sample. Variations in the Raman peak frequency reflect the stress or strain conditions of the sample's molecules. Additionally, the orientation and symmetry of the sample's crystal structure can be inferred from the polarization of the Raman peak [24].

After physical and chemical characterization, PLGA nanoparticles of *A. paniculata* ethanol extract were then tested for toxicity *in vitro* in Raw 264.7 cells using MTT assay. The results of the toxicity assay can be seen in fig. 5.

Based on the results of the cell viability test, up to a concentration of 100 ppm, the *A. paniculata* ethanol extract nanoparticles have not shown toxicity, although there was a decrease in cell viability as the concentration of PLGA-*A. paniculata* ethanol extract nanoparticles increased. Nanoparticles are toxic to normal cells if the cell viability is equal to or less than 50% [25]. The research results of Bardi *et al.* [26] stated that *A. paniculata* leaf extract prevents thioacetamide-induced liver cirrhosis in rats at the dose of 500 mg/kg BW. In future research, it is necessary to test the toxicity of the ethanol extract of *A. paniculata*-PLGA nanoparticles until the concentration is equivalent to 500 mg/kg BW. The MTT assay has been widely used to test the

cellular cytotoxicity of many drugs and other biological compounds on various types of cells, including Raw 264.7 macrophage cells. However, the results of the MTT test are greatly influenced by the number of cells, MTT concentration, and MTT incubation time [17].

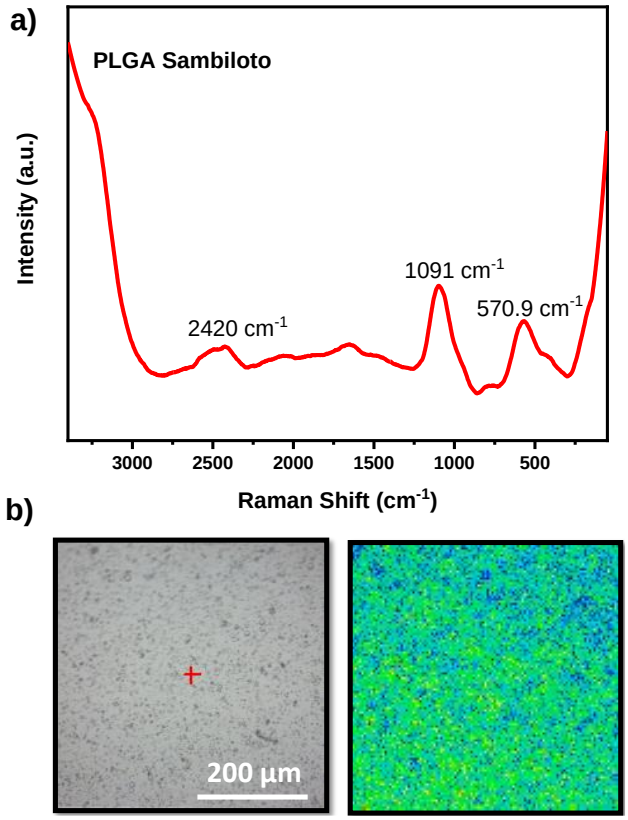

Fig. 4: The images of Raman spectra. a) Raman spectra vibration; b) The extrapolation of nanoparticles distribution

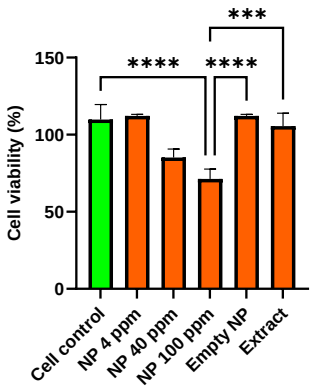

Fig. 5: Cell viability of Raw cells 264.7. Data are presented as mean±SD. *p<0.05, **p<0.01 and ***p<0.001 denotes significance

CONCLUSION

Based on the research results obtained, it can be concluded that *A. paniculata* ethanol extract nanoparticles have good characteristics in terms of particle size, particle size distribution, and morphology. The results of analysis using FTIR and Raman spectroscopy showed that the ethanol extract of *A. paniculata*, which in this study was also proven to contain andrographolide, was loaded in PLGA nanoparticles. The nanoparticles were proven to have no toxicity *in vitro* assay using the MTT test on Raw macrophage 264.7 cells.

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AUTHORS CONTRIBUTIONS

DWK: Conceived, designed, and performed the experiments, data analysis, writing, and finalized the manuscript. NSAG: data analysis, reviewed the manuscript. ARR: data analysis, writing and reviewing the manuscript. SH: performed the research data analysed, wrote manuscript draft. DN: data analysed, reviewed, and finalized the manuscript draft. TA: reviewed and finalized the manuscript.

CONFLICT OF INTERESTS

There is no conflict of interest among the authors.

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