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**Original Article**

# Moringa oil-based nanocarrier system containing curcumin formulation as anti-breast cancer agent: Efficacy and safety study

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## Abstract

Current anti-breast cancer drugs have limited efficacy and often cause severe side effects, highlighting the need for bioactive agents that could overcome these limitations. Curcumin, a phenolic compound from *Curcuma domestica*, has antineoplastic activity but has low solubility in physiological media, while moringa oil is a key component of the oil-phase nanocarrier and also possesses anticancer properties. The aim of this study was to develop a moringa oil-based nanocarrier system containing curcumin and to analyze its anticancer effects on MDA-MB-231 cell lines, focusing on the underlying mechanisms involving B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X (Bax) proteins. Additionally, the study investigated the side effects of the nanocarrier system following acute administration in animals. The anticancer effects were evaluated in vitro using MDA-MB-231 cell lines, while the acute toxicity assessment was conducted in healthy female Wistar rats. The nanocarrier system was formulated using moringa oil, Cremophor RH40, and PEG 400. Its cytotoxicity against MDA-MB-231 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. DNA fragmentation, apoptosis, and the expression of Bax and Bcl-2 proteins were analyzed via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays, flow cytometry, and western blotting. Acute toxicity was further evaluated in female Wistar rats. The results demonstrated that the moringa oil-based nanocarrier system containing curcumin inhibited cell proliferation and induced apoptosis in MDA-MB-231 cells. Curcumin suppressed tumorigenesis by modulating Bcl-2 and Bax protein expression. Our data indicated that the combination of curcumin and moringa oil in a nanocarrier system had greater anticancer potential than either component alone. Moreover, administration of the nanocarrier system did not result in any clinically significant changes in body weight, behavior, or organ weight indicative of toxicological effects. No treatment-related histopathological abnormalities were observed at terminal necropsy. In conclusion, this novel combination of curcumin and moringa in nanocarrier system has better anticancer potential; nevertheless, further studies are needed to confirm this in cancer animal models.

**Keywords**: Curcumin, moringa, nanocarrier, MDA-MB-231, toxicity

## Introduction

Breast cancer is the sixth most common cause of cancer-related deaths globally, accounting for an anticipated 685,000 deaths among women in 2020 [1]. Breast cancer was the most common cancer diagnosed in women, with a prevalence of 11.7% among all cancer cases in 2020 [1].



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Developed countries had an 88% higher incidence rate of breast cancer than underdeveloped countries (55.9 vs 29.7 per 100,000) [1]. Compared to developed countries, the mortality rate from female breast cancer was 17% higher in developing countries (15.0 vs 12.8 per 100,000) [1]. However, out of 396,914 new cases of cancer in Indonesia in 2020, 68,858 cases (16.6%) were breast cancer, and 22,430 fatalities were reported [1]. The treatment of breast cancer requires a combination of various approaches, including surgery, radiotherapy, chemotherapy, hormonal therapy, and biological therapies, administered in different sequences [2]. These therapies may stop cancer from growing and spreading; however, cancer treatment that involves radiation, chemotherapy, or both tends to change the body's defenses, reducing immunity and ultimately leading to death [3]. Many chemotherapeutic agents, such as doxorubicin, have been shown to have adverse side effects [4]. The side effects of doxorubicin may present in a matter of days (acute toxicity) or even years (chronic toxicity) following chemotherapeutic treatment [4]. Therefore, it is necessary to develop alternative medical therapies that can overcome the side effects that occur, such as herbal medicine, for example curcumin and moringa (oil). Herbal medicine has been proven to be useful in treating various diseases, including cancer [4].

Natural medicines derived from medicinal plants have been utilized to cure a variety of illnesses. New treatments employing natural materials such as curcumin and moringa have gained importance as the majority of target-specific anticancer medications did not produce the expected results [5]. Curcumin, a yellow polyphenolic compound from the rhizomes of *Curcuma domestica*, is widely used in Ayurveda and Chinese medicine. Curcumin has been widely used in traditional medicine due to its pharmacological effects, such as antioxidant, anticancer, anti-inflammatory, and antimicrobial activities [6]. Curcumin exerts anticancer properties in breast cancer, colon cancer, prostate cancer, leukemia, lymphoma, hepatocellular carcinoma, basal cell carcinoma, neuroblastoma, melanoma, liver, lung, and kidney carcinoma [1-2]. The anticancer mechanism of curcumin interferes with all major stages of carcinogenesis, including cell proliferation, growth, survival, angiogenesis, and metastasis through effects on cellular and molecular processes such as apoptosis, autophagy, Wnt/β-catenin, signal transducers, and activators of transcription processes and nuclear factor-kappa B (NF-κB) signaling [8-11]. However, curcumin has unfavorable properties among its many benefits, such as high lipophilicity and poor bioavailability after oral administration [12]. Therefore, many efforts have been made to increase the value of curcumin, including formulating curcumin into a spontaneous nanoemulsion system. Previous studies have shown significant improvements in curcumin's physical properties, bioavailability, and stability in nanoemulsion carrier systems [12-16].

As previously reported, approximately 74% of known anticancer drugs are sourced from various plant species [17]. Apart from curcumin, there are other plants that have anticancer potential, *Moringa oleifera*, which can potentially act as an anticancer agent against several types of cancer. *M. oleifera* leaf extract has been shown to inhibit the survival of hepatocellular carcinoma cells and acute lymphoblastic and myeloid leukemia cells [18]. *M. oleifera* leaves inhibited pancreatic cancer cell growth by targeting the cell cycle, resulting in the accumulation of cells in the sub-G1 phase [19]. *M. oleifera* also down-regulated the NF-κB pathway by decreasing the expression of inhibitor of NF-κB inhibitor α (IκBα), phosphorylated form of IκBα (pIκBα) and p65 proteins [19]. It synergistically induced cytotoxicity with cisplatin in pancreatic cancer cells and was also effective against breast cancer cells [20]. The anticancer properties of moringa are attributed to polyphenols such as flavonoids, tannins, and terpenoids [21]. Although polyphenols exhibit antineoplastic properties, their hydrophilic nature results in poor permeability across the plasma membrane and limited lipid solubility, restricting their uptake into target cells and consequently reducing their bioavailability and therapeutic effectiveness [21]. *M. oleifera* polyphenols (Mopp) undergo degradation by digestive enzymes and gastric juices when taken orally [21].

Since both curcumin and moringa have similar effects on breast cancer, the combination of the two would be more potent. However, there are some drawbacks that can reduce the effectiveness of both. A strategy to reduce the drawbacks could be by developing a nanocarrier, as moringa oil can act as an oil phase and play a role in increasing the anticancer effectiveness of curcumin. Research in cancer therapy is becoming more focused on nanoemulsion carrier systems because nanoemulsion carrier system has important characteristics that help achieve

efficient therapeutic effects: large surface area, superficial charge, increased circulating half-life, specific targeting, and imaging capacity of the formulation [22]. Nanoemulsion carrier systems are colloidal dispersions that can be used as drug carriers, mainly for molecules with low water solubility that are safe-grade excipients. This nanoemulsion carrier form consists of a heterogeneous dispersion of nanometer droplets in aqueous phase, which results in high stability and solubility. Nanoemulsion encapsulation is a technique that uses nanoemulsion to coat substances at the nanoscale. Encapsulation protects the substances or drug from degradation and increases its half-life in plasma [23].

Since cancer cells are surrounded by vascularized tissues, nanoemulsion can easily accumulate in these tissues, with their size as an advantage to cross the barrier. Moreover, they can also be designed to define their function, encapsulate different types of drugs, and select specific targets [23]. In this study, the nanoemulsion carrier system was developed using curcumin as the main drug and moringa oil as the oil phase. The aim is to improve the efficacy of both active components. The potential effect of the nanocarrier system containing curcumin as an anti-breast cancer was evaluated in vitro using breast cancer model cell: MDA-MB-231, with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, as well as apoptosis assay by flow cytometry and western blot analysis. The study also investigated the side effects of nanocarrier system after acute administration in rats. In the end, this nanoform is expected to show promise as an alternative therapeutic agent in breast cancer therapy while maintaining the safety.

# Methods

## Study design and setting

The nanocarrier of the curcumin using moringa oil as an oil phase was prepared and characterized. Then, an in vitro experimental study was conducted with a post-test-only design using human MDA-MB-231 breast cancer cell lines. Viability assay procedure using MTS method was conducted at the Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor, Indonesia, while other procedures were carried out at the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. The inhibition concentration at 50% ($IC_{50}$) value of curcumin-moringa nanocarrier obtained from viability assay was utilized as baseline dose to evaluate their effects on apoptosis of MDA-MB-231 cells. The study had five groups: control, curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, and doxorubicin. Cell death detection was conducted to assess the ability of curcumin-moringa nanocarrier to increase apoptosis cell death. Apoptosis cell death was detected using the TUNEL assay. Following a 72-hour exposure period, apoptosis levels were measured using flow cytometry and the synergistic effect was determined based on the viability assay of MDA-MB-231 cells. Western blot analysis was performed to elucidate the mechanism by which B-cell lymphoma 2 (Bcl-2) as the anti-proliferative and Bcl-2-associated X (Bax) as pro-apoptotic pathways were induced. The overall study was described in detail as presented in **Figure 1**. The side effect of nanocarrier system was also assessed after acute administration in rats.

## Preparation of curcumin-moringa nanocarrier system

A nanocarrier system was made using moringa oil as an oil phase, Cremophor RH40 (Merck, Darmstadt, Germany) as the surfactant, and polyethylene glycol (PEG) 400 (Merck, Darmstadt, Germany) as a co-surfactant was combined at a ratio of 1:8:1. Curcumin was purchased from PT. Phytochemindo Reksa (Bogor, Indonesia), while moringa seed oil was obtained from PT. Darjeeling Sembrani Aroma (Bandung, Indonesia). Curcumin, at a concentration of 2% weight/volume, was introduced into a mixture of moringa oil, Cremophor RH40, and PEG 400 while stirring with magnetic stirrer at 200 rpm for two hours. Additionally, the oil phase was immersed in an ultrasonic bath (Krisbow, Jakarta, Indonesia) for one hour at a temperature of 25°C in order to finalize the process. Afterward, the product was introduced into deionized water in a 5:1 ratio and mixed with gentle agitation for 15 minutes at 200 rpm until a transparent and uniform system was achieved [10]. As a comparison to the curcumin nanocarrier in moringa oil

as the oil phase, other curcumin nanocarriers were made using oil phases other than moringa, with a composition consisting of castor oil, Cremophor RH40, and PEG 400 in a ratio of 1:8:1. Additionally, moringa oil nanocarrier (without curcumin), curcumin dispersion and moringa oil were prepared.

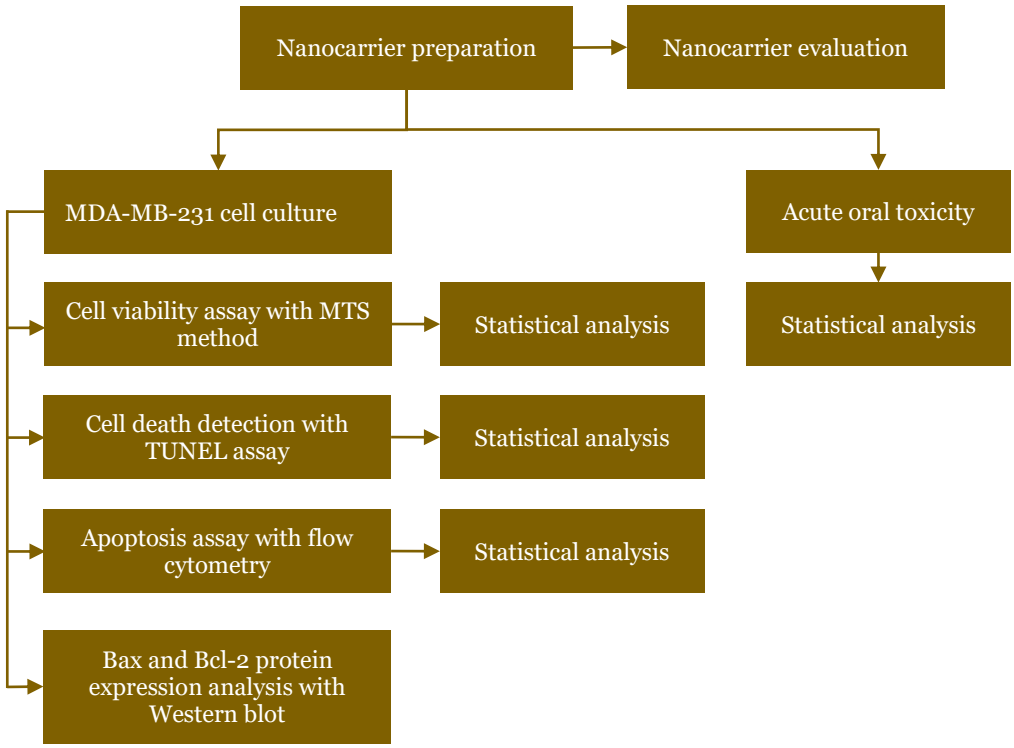


Figure 1. Flowchart of the study.

## Evaluation of curcumin-moringa nanocarrier system

The particle size and size distribution of the nanocarrier were assessed using the Delsa Nano C Particle Analyzer (Beckman Coulter, California, USA) by photon correlation spectroscopy. The zeta potential of curcumin-moringa nanocarrier was determined using a nanoparticle analyzer (Nano partica, Horiba Scientific SZ-100, Kyoto, Japan) by the electrophoretic light scattering method. The curcumin-moringa nanocarrier was positioned within an electrophoretic chamber and maintained at a consistent temperature of 25°C [24]. To determine the curcumin content that can be entrapped in the nanocarrier system, a loading capacity test was carried out using a T60 UV-visible spectrophotometer (PG instruments, Leicestershire, United Kingdom). Curcumin content in curcumin-moringa nanocarrier was determined using a direct method, as previously reported [25]. Briefly, the nanocarrier was centrifuged at 14,000 rpm for 20 min to break up the nanocarrier and 5 mL of DMSO was then added to 10 µL of supernatant to extract the curcumin. The curcumin content in the DMSO was quantified using a UV-visible spectrophotometer using the following equation: percentage of the loading capacity is equal to the measured amount of curcumin encapsulated in nanocarrier divided by the total amount of curcumin added during nanocarrier preparation, multiplied by 100%.

## Cell culture

Breast cancer cells (MDA-MB-231), triple-negative human breast cancer cells, were obtained from the Cell Biology and Molecular Laboratory, Pharmacy of Faculty, Universitas Padjadjaran, Indonesia. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum and 1% penicillin and streptomycin (all from Sigma, St Loius, USA), and maintained at a 37°C incubator with 5% $CO_2$.

### Cell viability assay and $IC_{50}$ measurement

Cell viability assay was done to assess curcumin-moringa nanocarrier capability on decreasing MDA-MB-231 cell proliferation and its $IC_{50}$ dose. The cell viability assay of the curcumin-moringa nanocarrier was evaluated against triple-negative breast carcinoma (MDA-MB-231, ATCC HTB-26) cell lines using the MTS method conducted according to the manufacture protocol (Dojindo Molecular Technologies, Kumamoto, Japan). Doxorubicin was used as a reference drug. Curcumin, moringa oil, curcumin nanocarrier, and moringa nanocarrier were also evaluated as comparison groups on the tests against MDA MB-231 cell lines. MDA-MB-231 cells suspension were placed in 96-well plates with 4000-5000 cell density per well and pre-incubated for 24 hours at 37°C. Subsequently, various doses of curcumin-moringa nanocarrier (10, 20, 40, 80, and 100 ppm), curcumin nanocarrier (10, 20, 40, 80, and 100 ppm), curcumin (10, 20, 40, 80, and 100 ppm), moringa nanocarrier, moringa oil, and doxorubicin (0.38, 0.75, 1.50, 3.00, and 6.00 ppm) were administered to the cell plates for 48 hours. The cells were mixed with the Cell Counting Kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan), by adding 10 µL CCK-8 and incubated at 37°C for four hours. The cell culture's relative absorbance was measured using a TECAN Infinite M200 Pro microplate reader (Tecan Trading AG, Mannedorf, Switzerland) with a wavelength of 450 nm. The assay detects the reduction of the MTS tetrazolium compound by metabolically active cells, generating a colored formazan dye that is soluble in cell culture media [26].

### Cell death detection

Apoptosis is a programmed form of cell death that eliminates damaged, unneeded, or stressed cells. As more cells undergo apoptosis, the overall viable cell population decreases. One of the characteristics of apoptosis is DNA fragmentation. Cell death detection was conducted to assess the ability of curcumin-moringa nanocarrier to increase apoptosis cell death. Apoptosis cell death was detected using the TUNEL assay. TUNEL assay was assessed using an In Situ Cell Death Detection Kit AP (Roche, Mannheim, Germany) following the manufacturer's instruction. Briefly, MDA-MB-231 breast cancer cell lines ($5\times10^4$) were cultured on 15-well plates and incubated for 24 hours. The cells were then treated with curcumin-moringa nanocarrier $IC_{50}$ value (9.04 ppm), curcumin nanocarrier (9.04 ppm), and doxorubicin (0.25 ppm). Moringa nanocarrier used the same volume as the volume of curcumin-moringa nanocarrier. Next, the cells were washed with PBS and fixed in a paraformaldehyde solution [27]. The cells were incubated in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for one hour at 4°C, and then treated with solutions A and B for one hour in the dark at 37°C. Analysis was performed by fluorescence microscopy using a laser scanning confocal imaging system.

### Quantitative measurement of cell apoptosis

To analyze whether curcumin-moringa nanocarrier has a role in regulating MDA-MB-231 cell death, a cell apoptosis assay was conducted. The apoptosis assay consisted of five groups: one control group (untreated cells), one group with treated doxorubicin, and three treatment groups consisting of curcumin-moringa nanocarrier, curcumin nanocarrier, and moringa nanocarrier with a concentration of 9.04 ppm each based on $IC_{50}$ value obtained from the previous viability assay. Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Thermo Fisher Scientific, Massachusetts, USA). Briefly, The MDA-MB-231 cells were washed twice with cold BioLegend's Cell Staining Buffer (Elabsciences, Houston, TX, USA) before being suspended in Annexin V Binding Buffer (Elabsciences, Houston, TX, USA) at a concentration of $1.0\times10^5$ cells/mL. Then, 100 µL of cell suspension was transferred into a 5 mL test tube, and 5 µL of FITC Annexin V (Elabsciences, Houston, TX, USA) and 10 µL of propidium iodide solution (Elabsciences, Houston, TX, USA) were added, then gently vortexed. Subsequently, the cells were incubated for 15 mins at room temperature (25°C) in the dark. Eventually, 400 µL of Annexin V Binding Buffer was added to each tube. The cell apoptosis was analyzed by Becton Dickinson (BD) FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). The apoptosis rate (%) was calculated as the percentage of cells in the lower right quadrant (early apoptosis cells) and upper right quadrant (late apoptosis cells) in the scatter diagram [28].

## Proteins expression analysis

Bcl-2 and Bax are crucial regulators of apoptosis, with Bcl-2 serving as an anti-apoptotic protein and Bax as a pro-apoptotic protein. The balance between these proteins is essential in determining cell survival or death. To investigate the effect of curcumin-moringa nanocarrier on Bcl-2 and Bax protein expression, which play key roles in regulating apoptosis, western blot was conducted. β-actin served as a loading control. MDA-MB-231 breast cancer cells were seeded ($5\times10^5$) for 24 hours, and the cells were then treated with curcumin-moringa nanocarrier ($IC_{50}$ value, 9.04 ppm), curcumin nanocarrier (9.04 pm), moringa nanocarrier (9.04 ppm), and doxorubicin (0.25 ppm). The cells were harvested by trypsinization and washed with PBS, and the pellet was resuspended in 20 µL of a protease inhibitor solution and 100 µl of cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1 mmol phenylmethylsulfonyl fluoride, 100 µM leupeptin, and 2 µg/L aprotinin).

## Acute toxicity study of curcumin-moringa nanocarrier

Curcumin has promising therapeutic benefits but is limited by poor bioavailability. Nanocarriers enhance curcumin's stability, solubility, and targeted delivery, potentially altering its toxicity profile. To assess potential toxicity of curcumin-moringa nanocarrier due to nanocarrier composition or interaction, an acute toxicity test was performed. A total of 15 female animals were randomly assigned to non-treated (normal control) group, curcumin-moringa nanocarrier treated group, and placebo treated group (five animals for each group). The single-dose acute oral toxicity of curcumin-moringa nanocarrier was tested according to OECD test guideline 425 for testing of chemicals [29], which elaborated a stepwise dose level method. The acute oral toxicity of curcumin-moringa nanocarrier was classified based on the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) 2003, in which the most severe toxicity was classified as category 1 (lethal dose 50 ($LD_{50}$) ≤0.005 g/kg body weight (BW)) and relatively low toxicity is classified as category 5 ($LD_{50}$ >2–5 g/kg BW). Agents that have very low toxicity ($LD_{50}$ >5 g/kg BW) [15] were placed into the unclassified hazards category as indicated by the ∞ symbol based on OECD guidelines [30]. Most previous articles have reported $LD_{50}$ of curcumin to be approximately 2 g/kg BW (in GHS category 5 of OECD guidelines for oral toxicity studies) in rats and mice. Therefore, in this study, the dose of 2 g/kg BW was used. All animals were fasted overnight prior to dosing and for three hours after treatment. Curcumin-moringa nanocarrier was administered orally as a single-dose at 2 g/kg BW. The rats in the placebo group received a single oral dose of blank nanocarrier, and the negative control group received no intervention. The weight changes of rats in each group were recorded. Clinical signs of toxidromes (food/water consumption, mucus secretion, color of fecal, diarrhea, sedation, eye color, convulsion, drowsiness, skin change, urination, and coma) and mortality were observed and recorded twice daily for 14 days post-treatment.

## Sample collection and histopathological study

Animals were euthanized by cardiac puncture. Internal organs, including the liver, lung, kidney, heart, stomach, and intestine, were collected and immediately fixed in 10% buffered formalin for histopathological study. The tissues were processed using a Tissue-Tek TEC 5 (Sakura Finetek, USA) and embedded with paraffin. The paraffin-embedded tissue was cut using a microtome and stained with hematoxylin and eosin (HE). The slides were observed under a light microscope.

## Statistical analysis

The results were presented as the means and standard deviation (SD). Nonlinear regression analysis was used to determine the $IC_{50}$ of curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, curcumin, and moringa oil for MDA-MB-231 cells. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to analyze the viability, the cell death, the cancer cell's apoptosis, and the toxicity against curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, curcumin, and moringa oil, respectively. Those with $p<0.05$ were regarded as statistically significant differences. All analyses were performed using SPSS 26.0 software (IBM, New York, USA). The $IC_{50}$ was calculated using linear regression analysis with GraphPad Prism 9 (GraphPad Software, California, USA).

# Results

## Characteristics of the curcumin-moringa nanocarrier system

The nanocarrier containing curcumin was successfully produced with an average globule size of 33.27±2.28 nm and had a negative charge of -7.78±0.49 mV. The polydispersity index value was 0.38±0.07, indicating a narrow distribution size. The nanocarrier formula containing the amount of curcumin produced curcumin-moringa nanocarrier with 98.79±0.62% of loading capacity.

## Cell viability and $IC_{50}$ values of curcumin-moringa nanocarrier, curcumin nanocarrier, and moringa nanocarrier for MDA-MB-231 cell lines

The cell viability was significantly lower with increasing concentrations of curcumin-moringa nanocarrier in a dose-dependent manner (beginning from 20 ppm, $p<0.05$) (**Figure 1A**). The viable cells decreased to 74.03±2.18% and 3.61±1.68% in curcumin nanocarrier group at 20 and 80 ppm, respectively. Meanwhile, the cell viability was recorded as 80.10±1.68% and 5.49±0.19% in moringa nanocarrier group, and 57.78±5.75% and 1.09±0.33% in curcumin-moringa nanocarrier group at 20 and 80 ppm concentrations, respectively, which were also lower when compared to curcumin and moringa treated group ($p<0.05$). However, in all concentrations, the results also demonstrated that curcumin-moringa nanocarrier inhibitory effect was much greater than that of the curcumin nanocarrier or moringa nanocarrier alone ($p<0.05$). Additionally, it was shown that the moringa nanocarrier did not affect the viability of cells. This finding suggested that the cytotoxicity results obtained with curcumin-moringa nanocarrier treatment were entirely due to the combination of curcumin and moringa in the nanocarrier system formulation. The $IC_{50}$ concentrations for curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, and doxorubicin were calculated as 9.04 ppm, 16.91 ppm, 17.42 ppm, and 0.94 ppm, respectively (**Figure 1B**).

## Effect of curcumin-moringa nanocarrier on DNA fragmentation during apoptosis of MDA-MB-231 cell lines

Curcumin-moringa nanocarrier dose on MDA-MB-231 cell lines was determined at 9.04 ppm, based on the previous $IC_{50}$ value results. Fluorescence staining was used to determine the changes in each group. The results indicated that the curcumin-moringa nanocarrier induced apoptotic cell death in MDA-MB-231 cells as presented by TUNEL assay in **Figure 2A**. The results indicated that the nuclear structure of control cells remained intact, whereas nuclear chromatin condensation and fragmentation, characteristics of apoptosis, were observed in cells treated with curcumin-moringa nanocarrier and doxorubicin as well (**Figure 2B**).

## Effect of curcumin-moringa nanocarrier on apoptosis of MDA-MB-231 cell lines

The MDA-MB-231 cells were treated with curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, and doxorubicin at $IC_{50}$ concentrations of curcumin-moringa nanocarrier and the cell populations coupled with annexin V-FITC were measured after a 72-hour incubation. In the flow cytometry results, after each treatment, the cells were divided into four groups presented in quadrants 1 to 4. The life cells were not labeled with PI or Annexin V; the early apoptotic cells were labeled only with Annexin V; the late apoptotic cells were labeled with both PI and Annexin V; and the dead cells were labeled only with PI. The results of flow cytometric analysis of apoptosis in MDA-MB-231 cells are presented in **Figure 3A**. The early and late apoptotic cell populations in **Figure 3B** were recorded as 4.46±1.10% and 2.23±0.94% in curcumin nanocarrier treated group, 3.42±0.55% and 1.04±0.19% in moringa nanocarrier treated group, and 7.91±0.54% and 1.11±0.26% in the control group, respectively ($p<0.05$). On the other hand, a significant increase in early and late apoptotic cell population was observed in curcumin-moringa nanocarrier treated group, recorded as 7.42±0.59% and 3.83±0.62%, respectively, when compared to the other group ($p<0.05$).

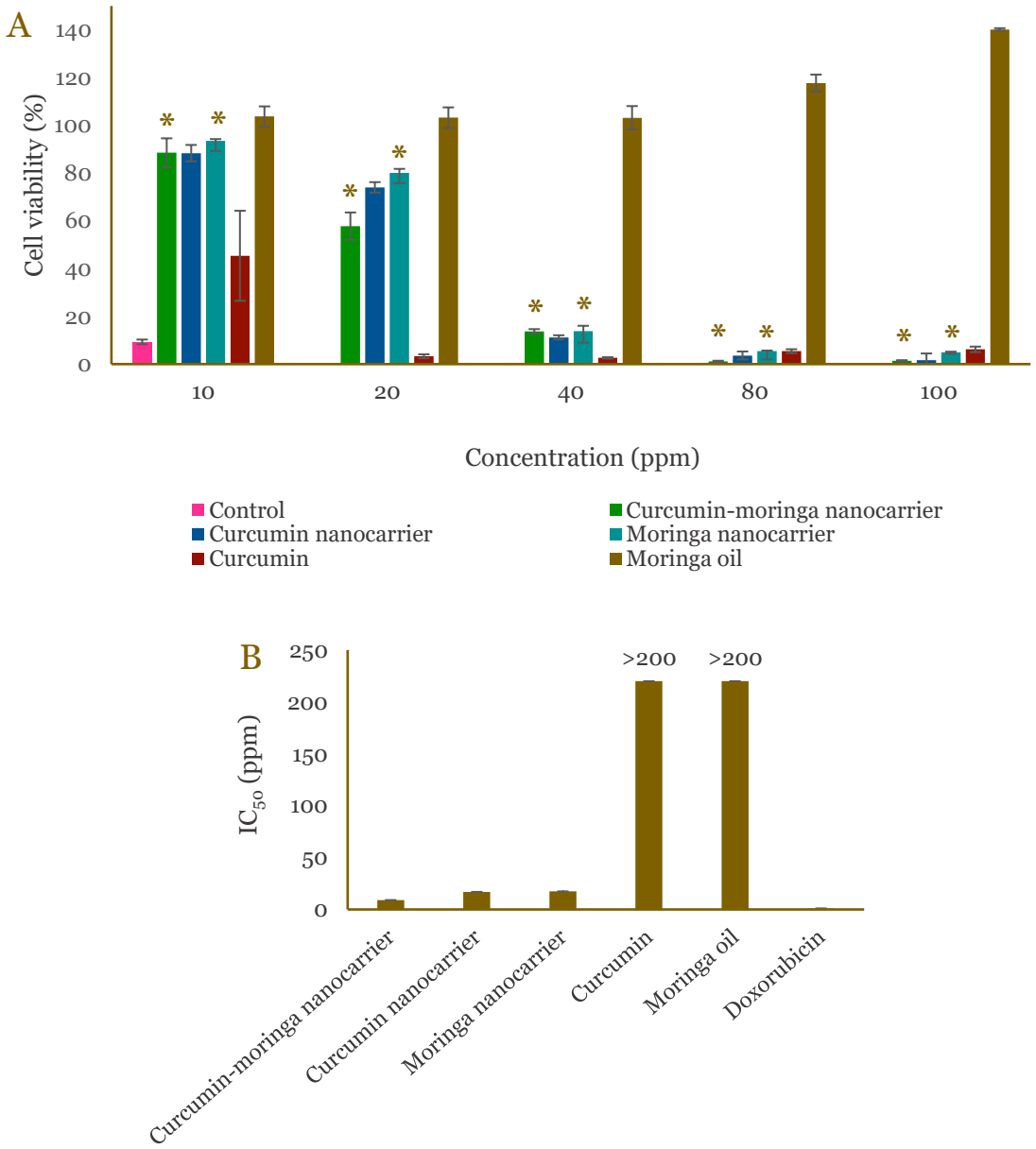


Figure 1. Cytotoxicity of the compounds against MDA-MB-231 human breast cancer cells. The cells were treated with curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, curcumin, and moringa oil at 10, 20, 40, 80, and 100 ppm concentrations for 24 hours, and the viable cell amount was determined by MTS assay (A). The results are expressed as percentage of live cells compared with untreated control. The cells were treated with curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, curcumin, and moringa oil with $IC_{50}$ values of 9.04, 16.91, 17.42, >200, >200 ppm, respectively for 24 hours and the cell population was detected by Infinite 200 Pro (Tecan Trading AG, Switzerland) (B). The data represent the mean ± standard deviation in three replicates. *Statistically significant differences at $p<0.05$.

## Involvement of Bcl-2 and Bax in curcumin-moringa nanocarrier-induced cell death

Since the Bcl-2 family members play a critical role in cell apoptosis, western blot analysis was performed to further elucidate the mechanism by which Bcl-2 as the anti-proliferative and Bax as pro-apoptotic pathways were induced. After treatment with curcumin-moringa nanocarrier, the expression of Bax was upregulated, whereas the expression of Bcl-2 was downregulated (**Figure 4**).

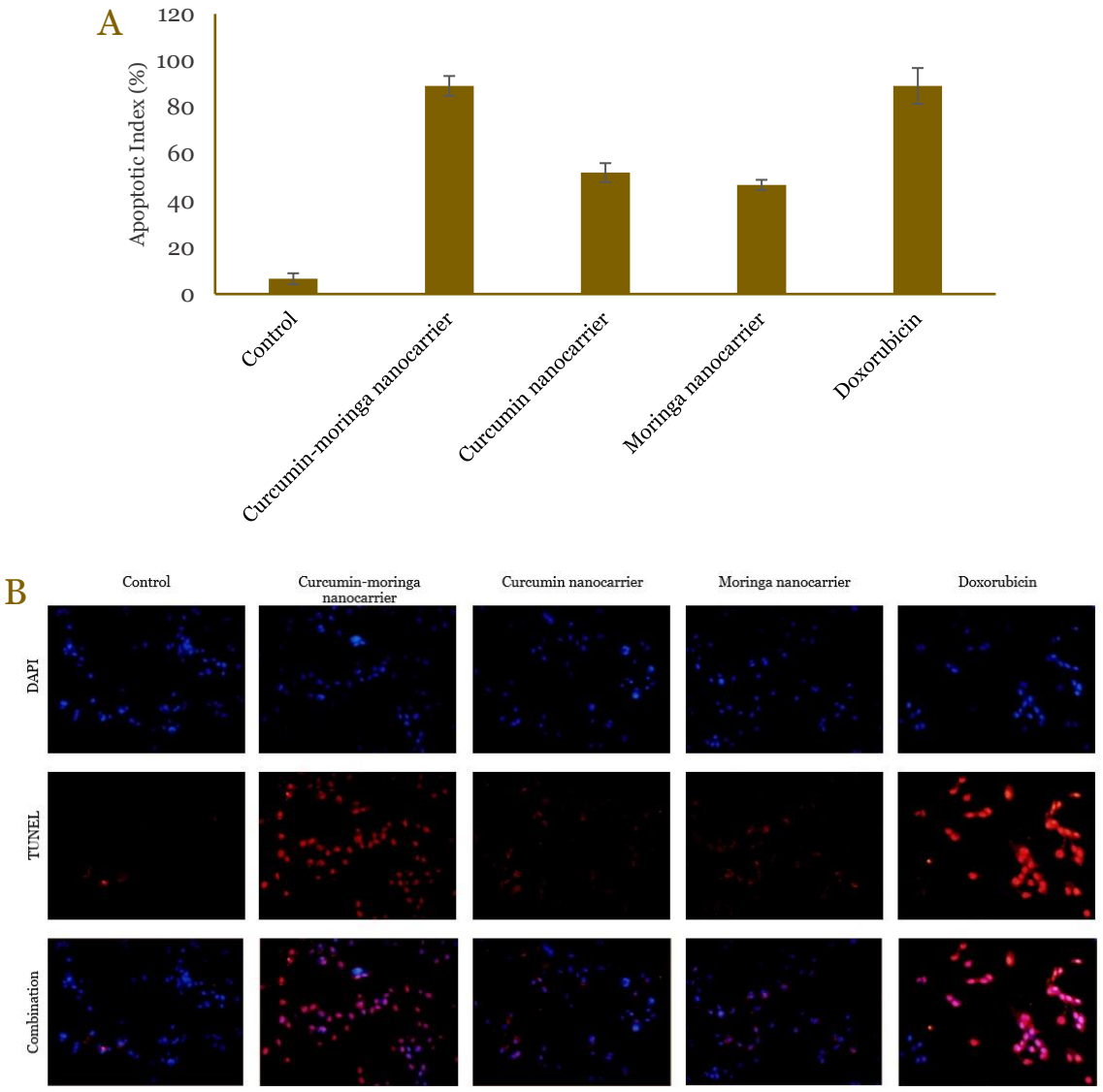


Figure 2. Effect of curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, and doxorubicin (as standard) in increasing apoptosis cell death. (A) The number of apoptotic cells was counted per 100 cells in each field of view. (B) Fluorescence imaging showing the effect of curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, and doxorubicin in apoptosis MDA-MB-231 cells. The images were obtained by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (4',6-diamidino-2-phenylindole (DAPI)) staining for nuclei (blue fluorescence) and TUNEL staining for apoptotic cells (red fluorescence) through fluorescence microscope (Olympus, Germany).

## Acute toxicity studies

A previous study [31] reported oral $LD_{50}$ of the curcumin to be approximately 2000 mg/kg BW in rats and mice, and this dose was examined in the acute toxicity test. According to body weight measurements, rats exhibited no harmful effects over the 14-day observation period of the acute oral toxicity investigation. The control group's initial mean body weight was 132.60 g and the mean body weight at the end of the treatment period was 158.00 g, suggesting normal body weight increase. Throughout the treatment period, there was no statistically significant change observed in the body weights of the animals in the treatment groups (curcumin-moringa nanocarrier and placebo) compared to the control group (**Figure 5**). This suggested that curcumin-moringa nanocarrier had no biologically significant impacts on body weight or weight growth compared to the control group at dose levels of 2000 mg/kg. The food intake by rats treated with curcumin-moringa nanocarrier or placebo group was similar to that of the control group for female rats.

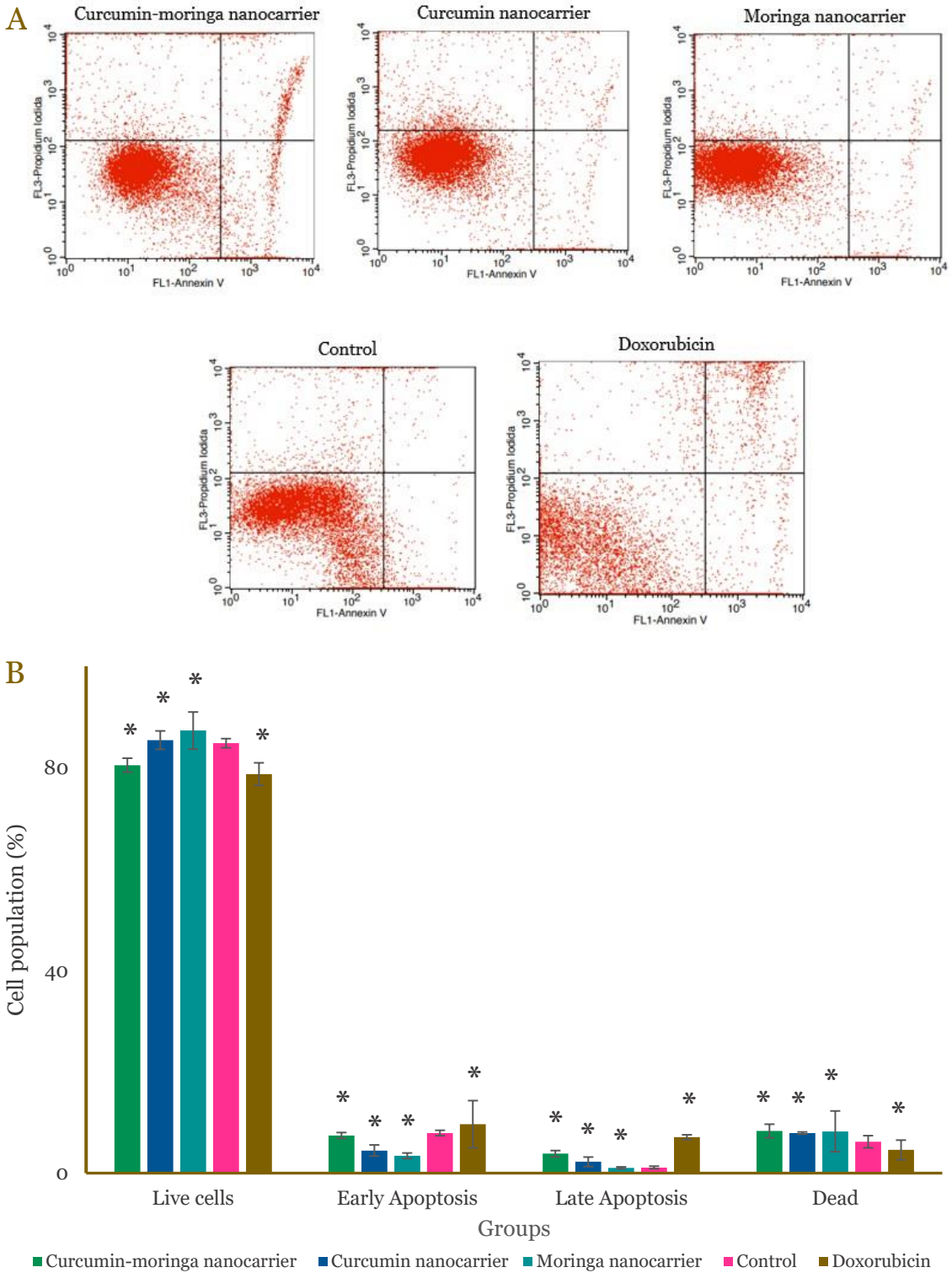


Figure 3. Effect of nanocarrier on apoptotic efficiency of the human breast cancer cells. (A) Flow cytometric analysis of apoptosis in MDA-MB-231. The cells were treated with curcumin-moringa nanocarrier, curcumin nanocarrier, and moringa nanocarrier with $IC_{50}$ values of 9.04, 16.91, and 17.42 ppm, respectively for 72 hours. Untreated cells and doxorubicin were used as control and standard drugs. (B) The apoptotic cell population percentage results from a FACScan flow cytometer on MDA-MB-231 cells using curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, control, and doxorubicin. *Statistically significant differences at $p<0.05$.

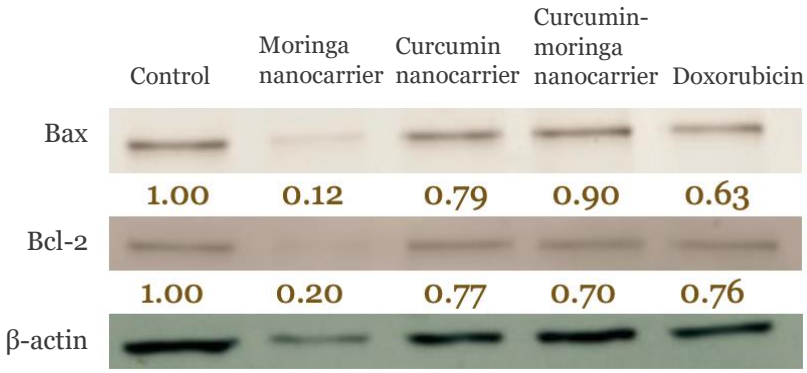


Figure 4. Effects of curcumin-moringa nanocarrier, curcumin nanocarrier, moringa nanocarrier, and doxorubicin on Bax and Bcl-2 proteins expression in MDA-MB-231 cells. β-actin served as a loading control.

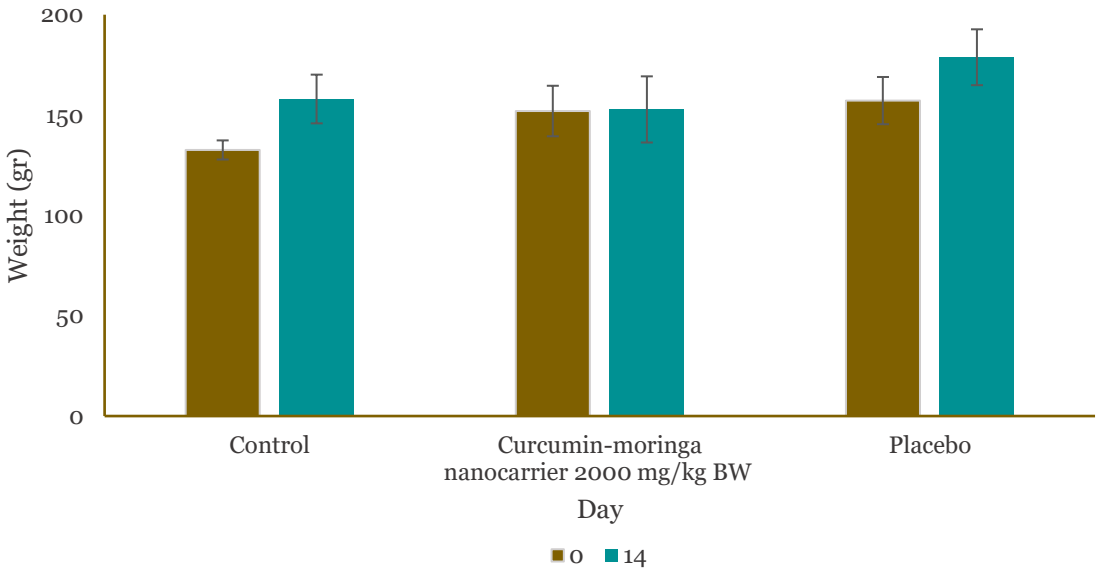


Figure 5. Body weight gain of rats during acute toxicity evaluation. There are no statistically significant differences in body weight between the groups administered with curcumin-moringa nanocarrier, placebo, and control group; analyzed using Tukey test in comparison with control.

At the end of the investigation, necropsy did not find any obvious pathological anomalies in the rats. Every animal in the treated and the control groups lived until the planned necropsy. None of the groups experienced aberrant clinical symptoms connected to their treatment throughout the trial period. Similarly, assessment of fecal color, eye color, and urination also did not reveal any significant differences between the groups. Meanwhile, all groups have negative mucus secretions, negative diarrhea, negative sedation, negative convulsion, negative drowsiness, negative skin change, and negative coma.

Regarding organ weights, administration of the curcumin formulation to rats did not result in biologically significant treatment-related changes in absolute organ weights (all had $p>0.05$ for all organs) (**Figure 6A**). At the planned necropsy, microscopic analysis revealed no treatment-related macroscopic findings in any group following curcumin-moringa nanocarrier administration. Additionally, no histological abnormalities unrelated to treatment were observed (**Figure 6B**).

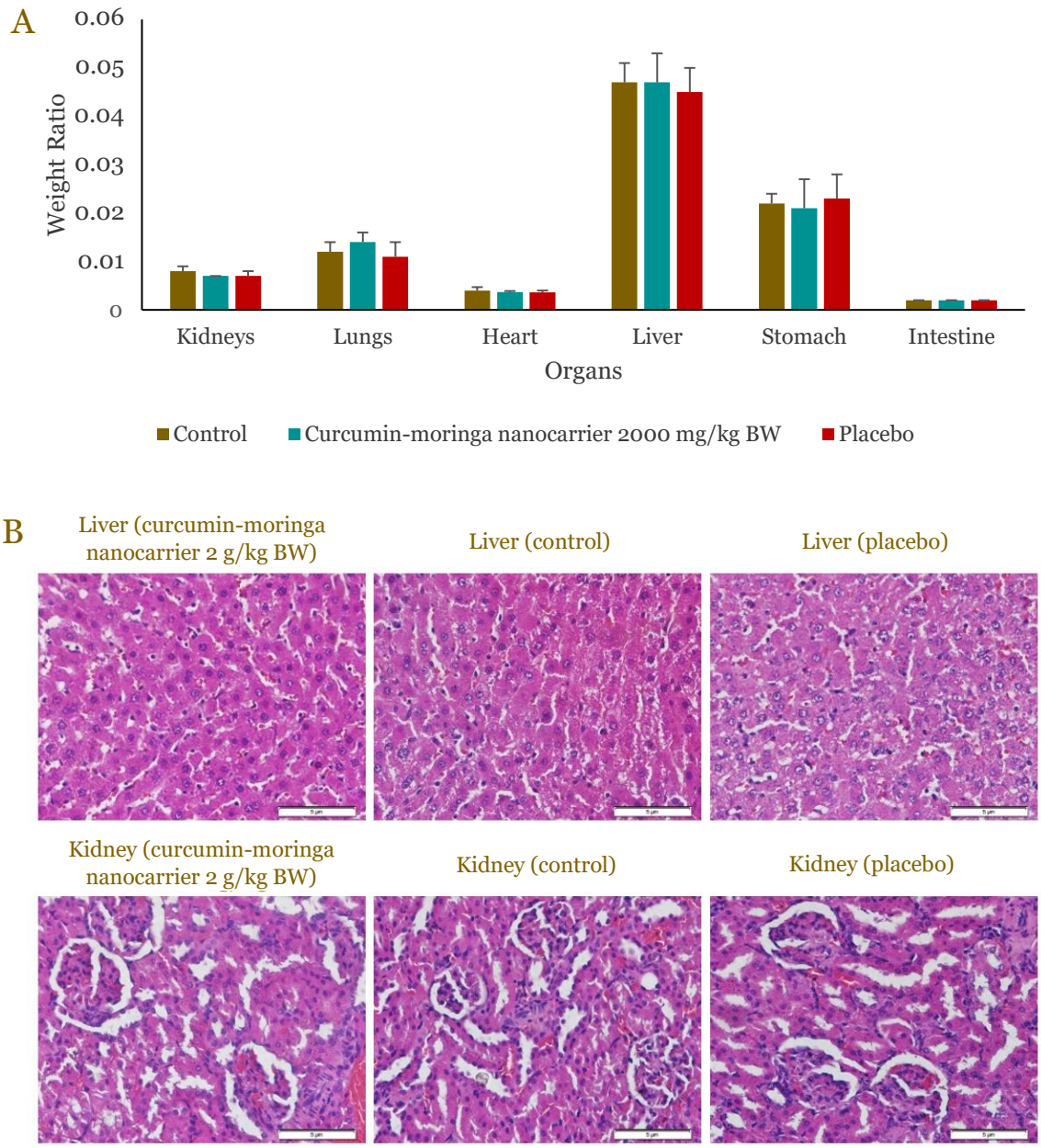


Figure 6. Organ evaluation for the acute toxicity experiments. (A) Comparison of the organ weight evaluation between control, curcumin-moringa nanocarrier, and placebo groups. There were no statistically significant differences in organ weight between the groups (Tukey test; $p>0.05$, compared with the respective control). (B) Representative images of the effect of curcumin-moringa nanocarrier in the histopathological parameters in liver and kidney. Staining with hematoxylin and eosin, 400× magnification.

## Discussion

Curcumin is a bioactive curcuminoid component derived from turmeric, and studies have shown that curcumin inhibited the growth of various human cancer cells and tumor xenografts [7,28,32-35]. In addition, *M. oleifera* as a traditional medicinal tree also shown great potential in complementary and alternative medicine for cancer treatment [17,20,24,35-37]. However, the practical use of curcumin in clinical settings is limited since it has low solubility, poor absorption, and is quickly metabolized and eliminated. Recent research has made significant progress in identifying several components and strategies that can enhance the bioavailability of curcumin [12]. The utilization of adjuvants, transporters, and nanoforms, together with other bioactive compounds, synthetic derivatives, and structural analogs of curcumin, has demonstrated

enhanced effectiveness and bioavailability [12]. This, in turn, expands the scope of curcumin's applications [12].

The present study was conducted to determine the anticancer efficacy of using curcumin encapsulated and co-administered with moringa oil (oil phase) as a nanocarrier system for oral administration. The evaluation focused on factors such as particle size, cell viability testing, apoptosis, Bax and Bcl-2 proteins involved in apoptosis, and acute toxicity testing after oral administration. Regarding on zeta potential characterization results of the curcumin-moringa nanocarrier system, the negative charge resulted from the utilization of Cremophor RH 40, a dissociated fatty acid ester known for its low toxicity and good biological compatibility. This compound can generate negatively charged free fatty acids. The presence of a significant concentration of surfactant in the formula facilitated the stability of the droplets by providing complete coverage of the droplet surfaces.

The Western blot analysis revealed alterations in apoptosis-related protein expression. MDA-MB-231 cells were treated for 24 hours with the curcumin-moringa nanocarrier, curcumin nanocarrier, and moringa nanocarrier. Treatment with the curcumin-moringa nanocarrier increased pro-apoptotic Bax levels while simultaneously reducing anti-apoptotic Bcl-2 expression (**Figure 4**). Bcl-2 levels were significantly lower in the curcumin-moringa nanocarrier-treated group compared to the curcumin nanocarrier group ($p<0.05$). The downregulation of Bcl-2 and the upregulation of Bax promote mitochondrial outer membrane permeabilization, leading to cytochrome c release and caspase activation. Caspases play a key role in the apoptotic process, as they are initially synthesized as inactive proteins and later cleaved into their active forms in response to apoptotic signals [38]. Bax protein promotes caspase activation, leading to a significant increase in caspase-3 and caspase-9 expression. These findings suggest that the mitochondrial pathway may be the primary molecular mechanism of apoptosis, as indicated by the activation of caspase-3 and caspase-9.

Given the prospective health benefits and historical applications of turmeric, significant research has been conducted to explore the biological functions and mechanisms of action of curcumin, its active component [39]. Numerous preclinical studies, both in vitro and in vivo, have investigated the effects of curcumin and turmeric and more than 40 clinical studies have been completed, with 30 ongoing phase I and II trials assessing the efficacy of curcumin primarily focused on their antioxidant, anti-inflammatory, anti-cancer, and anti-atherogenic properties [40]. Several studies also evaluated both effectiveness and potential adverse effects in clinical observations, providing insight into the safety and tolerability of curcumin and turmeric extract [40]. Findings from clinical trials assessing safety indicate that oral administration of curcumin at doses up to 12 g/day for three months was well tolerated and did not result in any adverse effects and no dose-limiting toxicity was reported [39-41]. The present study on acute toxicity further confirmed the safety of curcumin, as the novel curcumin formulation, when administered to rats at 2000 mg/kg.

There are some limitations of the study. This study mainly focused on in vitro (cell lines) experiments to assess the efficacy as well as acute toxicity of the curcumin-moringa-based nanocarrier system. Although these results provide valuable insights into the potential anti-breast cancer effects, they may not fully describe the behaviour of the system in living organisms. Further in vivo studies are needed to confirm the efficacy in real biological environments. In spite of the fact that preliminary safety studies have been conducted, these studies are limited by lack of long-term toxicological evaluations. Chronic exposure and potential long-term adverse effects have not been thoroughly evaluated.

## Conclusion

The nanocarrier system combining curcumin and moringa oil significantly influenced both intrinsic and extrinsic apoptotic pathways, primarily by inhibiting Bcl-2 and Bax protein activity. When administered as a single dose, the curcumin-moringa nanocarrier demonstrated safety in rats, with no observed toxicity at the tested doses. Cytotoxicity and acute toxicity assessments confirmed that the nanocarrier does not pose immediate toxic risks. These findings suggest that the curcumin-moringa nanocarrier holds promise as a

potential breast cancer treatment. However, further research is needed to fully elucidate its molecular mechanisms and to evaluate its chronic toxicity at higher doses.

### Ethics approval

All of the experimental procedures involving animals were conducted following the Institutional Animal Care guidelines and approved by Universitas Airlangga Health Research Ethical Clearance Commission, Indonesia, Number: 399/HRECC.FODM/IV/2023.

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### Competing interests

All the authors declare that there are no conflicts of interest.

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### Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request. Final decisions and interpretations presented in this article were solely made by the authors.

### Declaration of artificial intelligence use

This study used artificial intelligence (AI) tools in manuscript writing support, of which AI-based language models, ChatGPT and Quillbot, were employed for language refinement (improving grammar and sentence structure). We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results. The final decisions and interpretations presented in this article were solely made by the authors.

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