

Research Article



Anti-Inflammatory and Anti-Cancer Activity of Moringa Leaf Extract Nanocrystal on MCF-7 Cells *In Vitro*

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ARTICLE INFO

Article history:

Received September 16, 2025

Received in revised form May 14, 2026

Accepted September 19, 2026

Available October 19, 2026

KEYWORDS:

anti-cancer,
anti-inflammatory,
nanocrystals,
MCF-7,
Moringa leaves

ABSTRACT

Moringa leaves are known as a rich source of antioxidants and are widely used as anti-diabetic, anti-inflammatory, and anti-cancer agents. Breast cancer is the most commonly diagnosed cancer worldwide, with the number of cases increasing each year. One of the side effects of current breast cancer therapies is the production of high levels of oxidative stress. The use of herbal materials such as moringa leaves is considered more effective because of their high content of bioactive compounds as anti-cancer agents without causing harm to normal cells. However, their application is limited by low bioavailability. Therefore, moringa leaf extract was prepared in the form of nanocrystals (NcML) using the top-down method to enhance its bioavailability as an anti-cancer and anti-inflammatory agent. The anti-cancer activity was assessed by cytotoxicity, senescence, and cell cycle distribution in MCF-7 cells. NcML was toxic to 11% of MCF-7 cells at 1 mg/mL and induced senescence up to 76.05%, with cell cycle arrest at the G2/M phase. The anti-inflammatory activity was observed by antioxidant activity, showing 43.68% inhibition in the DPPH assay and 85.65% inhibition of BSA denaturation at 4 mg/mL. Thus, moringa leaf extract in nanocrystal form exhibits anti-cancer and anti-inflammatory activities with potential therapeutic benefits.



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1. Introduction

The use of plants as alternative medicine has become commonplace, with the abundant and diverse compounds in plants leading the public to believe that some plants have benefits for treating certain diseases (Ghosh 2013; Tiloke *et al.* 2018). One of the diseases that has garnered attention in the search for alternative plant-based medicines is breast cancer. Breast cancer is the most diagnosed cancer disease worldwide and

the number of cases has been increasing every year. In 2020, there were 2.3 billion women diagnosed with breast cancer, with a mortality rate of 6.9%, and in 2022, the number of women diagnosed with breast cancer increased to 2.5 billion worldwide (Sung *et al.* 2021). For breast cancer, extensive research has been conducted on several plants that have the potential as alternative treatments by inhibiting metastasis, cell growth, proliferation, and enhancing apoptosis, such as turmeric, green tea, soybeans, apples, onions, chili peppers, watermelon, and tomatoes (Prakobwong *et al.* 2011; Fang *et al.* 2015; Varinska *et al.* 2015; Ceci *et al.* 2018; Lotfi *et al.* 2023). This is due to several cases of

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side effects from breast cancer treatments and therapies that can cause new problems such as increased risk of heart disease, osteoporosis, inflammation, autoimmune diseases, and depression. Even chemotherapy and radiotherapy can potentially lead to new tumors and damage to other tissues (Bodai & Tusso 2015; Shaik *et al.* 2022).

The side effects of the treatment are caused by the high levels of oxidative stress production because, fundamentally, the success of cancer therapies such as radiotherapy, chemotherapy, and immunotherapy is influenced by the inflammation occurring in the tumor microenvironment (TME) (Zhang & Jimenez 2023; Zhao *et al.* 2021). On the other hand, this inflammation also affects the development and malignancy of the tumor itself; it is known that 25% of cancer diseases are associated with chronic inflammation, such as colorectal cancer (Zhao *et al.* 2021). To address this issue, moringa leaves have potential as an alternative for treating breast cancer. Besides being known as a material rich in antioxidants due to the presence of abundant phenolics and flavonoids (El-sherbiny *et al.* 2024), moringa leaves have been extensively researched *in vitro* on several types of cancer and can influence cell migration and angiogenesis. Moringa leaf extracts using various extraction methods also show potential as preventive and even therapeutic agents for breast cancer cells such as MCF-7 and MDA-MB-231, with glucosinolates, niazimicin, and benzyl isothiocyanate identified as anticancer compounds (Ghosh 2013; Poobalan *et al.* 2018; Islam *et al.* 2021; Priyandoko *et al.* 2022).

However, one of the obstacles of treatment with plant extracts is low bioavailability, which affects the long distance traveled and can lead to a high required dose (Jahangir *et al.* 2022). Recently, there has been significant development of nanocrystal drugs for therapy on various cancer cells to enhance their bioavailability. For instance, research by Shete *et al.* (2022) showed that Hesperetin in nanocrystal form can release 46.3 µg/mL per minute in the body, compared to regular Hesperetin which can only release 29.5 µg/mL per minute, and this has been proven to increase ROS levels in MCF-7 cells. In addition to Shete *et al.* research, Fan *et al.* (2022) also stated that Paclitaxel in nanocrystal form can prevent the regrowth and metastasis of MCF-7 cells. Furthermore, research by Panwar *et al.* (2023) using MDA-MB 231 (epithelial breast cancer cells) showed that the IC₅₀ of Venetoclax, which was initially 19.49 µg/mL, became 3.96 µg/mL when in nanocrystal form (Chary *et al.* 2024).

Therefore, to determine the potential of moringa leaves as an anti-cancer and anti-inflammatory agent optimized in the form of nanocrystals, this research was conducted. Anticancer activity was conducted using cytotoxicity tests, senescence tests, and cell cycle assays on MCF-7. The anti-inflammatory parameters were measured using DPPH antioxidant activity tests and protein denaturation inhibition.

2. Materials and Methods

2.1. Nanocrystal Moringa Leaves (NcML) Preparation

The powdered moringa leaf extract is produced at PT Fathonah Amanah Shidiq Tabligh (FAST), Depok, West Java, Indonesia, which complies with Good Manufacturing Practice (GMP) standards, using a 70% ethanol extraction method with maceration. The nanocrystals were made at PT. Nanotech Global Indonesia (NANO) using the top-down method with a High Energy Milling Machine (HEMEllipse 3 Dimension).

2.2. Characterization of NcML

The physicochemical characteristics of NcML were evaluated by particle size and zeta potential analyses. Particle size was measured using a Zetasizer Pro (Malvern Panalytical), whereas zeta potential was determined using a nanoparticle analyzer (Horiba SZ-100). The analyses were performed at the Research Center for Nanoscience and Nanotechnology, Institut Teknologi Bandung, Bandung, Indonesia

2.3. MCF-7 Cell culture

The MCF-7 human breast adenocarcinoma cell line (ATCC HTB-22) was obtained from PT Aretha Medika Utama, Bandung, Indonesia. The exact passage number of the cells used in this study was not recorded. MCF-7 cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Biowest, L0103-500) supplemented with 15% fetal bovine serum (FBS; Gibco, 10270-106), 1% ABAM (Biowest, L0010-100), 1% amphotericin B (Biowest, L0009-050), 1% MEM vitamins (Biowest, X0556-100), L-glutamine (Biowest, X0550-100), 1% Nanomycopulitine (Biowest, LX16-100), and gentamicin (Gibco, 15750060). Cells were cultured in 25- or 75-cm² culture flasks and maintained at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was changed twice weekly until the cells reached the appropriate confluency. Cell

morphology and confluency were routinely monitored using an inverted microscope (Olympus CKX41-F32FL).

2.4. Anti-Cancer Assay

2.4.1. Cytotoxicity Assay

Cytotoxicity assay was conducted using the resazurin method at the Central Laboratory of Padjajaran University, Bandung Indonesia. MCF-7 cells were plated in a 96-well plate at a density 1×10^4 cells/well with 100 μ L consisting of 15% FBS (Gibco 10270-106), 1% ABAM (Biowest, L0010-100), 1% Amphotericin B (Biowest, L0009-050), 1% MEM vitamin (Biowest, X0556-100), L-Glutamine (Biowest, X0550-100), 1% nanomycopulitine (Biowest, L-X16-100), gentamicin (Gibco, 15750060), and high glucose DMEM basal medium (Biowest, L0103-500). After that, each well was added 100 μ L NcML, then incubated for 48 hours (at 37°C, 5% CO₂). Resazurin Sodium Salt-Powder (BioReagent) is added at a ratio of 9:1 after the medium is discarded and incubated again for 1-2 hours. After 1-2 hours, cell absorbance is measured at a wavelength of 570 nm (reference: 600 nm) using a multimode reader (Sianipar *et al.* 2020).

2.4.2. Senescence Assay

MCF-7 treated cells were subjected to senescence associated b-galactosidase (SA-b-gal) staining using the standard protocol Senescence Cell Histochemical Staining KIT (CS0030, Sigma). In brief, MCF-7 cells in 12-well plate with 10.000 cells density were first treated with NcML. The cells were washed with PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 10 min, and washed again with PBS, followed by an overnight incubation in staining solution. Stained cells were examined under the microscope and photodocumented.

2.4.3. Cell Cycle Assay

MCF-7 cells plated and about 50% confluent were treated with NcML for 24 and then harvested by trypsin. Cells pellets were washed with cold PBS and fixed in 70% ethanol at 4°C for 12 h. The fixed cells were centrifuged at 2000 rpm for 10 min, washed with cold PBS twice and then re-suspended in 0.25 ml PBS. The cells suspensions were prepared for cell cycle analysis followed by Cell Cycle Kit (E-CK-A351, Elabscience). MC7-cells suspensions treated with RNase A (5 ml, 1 mg/ml at 37°C for 1 h) to remove RNA followed by

incubation with Propidium Iodide (PI, 10 ml, 1 mg/ml) for 30 min in dark. PI-stained cells were subjected to cell cycle analysis using BD Acuri C6 Flow Cytometer (Beckman).

2.5. Antioxidant Activity Assay

Examination A DPPH solution of 6×10^{-5} M was prepared by dissolving 7.89 mg in methanol to a final volume of 100 mL. The NcML samples comprised five concentrations: 4, 2, 1, 0.5, and 0.25 mg/mL, dissolved in DMSO. This DPPH test utilized ascorbic acid as a comparator or positive control (PC) alongside the NcML samples, with concentration variations of 40, 20, 10, 5, and 2.5 ppm. Once all test materials are prepared, 500 μ L of the test solution (NcML, PC, NC) is combined with 500 μ L of DPPH at a concentration of 6×10^{-5} M in the test tube. The solution is homogenized for 30 minutes at a temperature of 25°C. Absorbance was quantified at 520 nm using a spectrophotometer, with methanol used as the blank. (HDT 2023).

2.6. Anti-inflammation Assay

The method used refers to the research by Saleem *et al.* (2020) using 0.5% BSA by weighing 0.05 g of BSA and then dissolving it using $1 \times$ PBS up to 10 mL. A total of 0.45 mL of 5% BSA was added to 0.05 mL of the sample solution, which consisted of NcML solution and a positive control (PC) consisting of 5 concentrations: 4, 2, 1, 0.5, and 0.25 mg/mL.

3. Results

3.1. Nanocrystal Moringa Leaf Extract Characterization

To observe the morphological changes from moringa leaf extract (ML) to nanocrystals of moringa leaf extract (NcML), characterization tests were conducted by examining the sizes of both, which can be seen in Table 1.

The particle size categories shown in Table 1, such as D10, D50, and D90, indicate the average particle size for 10%, 50%, and 90% of the measured samples. For example, for D90, the particle size of ML was 1136 nm, which then decreased to 323.2 nm after being transformed into nanocrystals or NcML. This means there was a reduction in the average particle size for 90% of the measured samples. The decrease in particle size due to transformation into nanocrystals is also evident in every particle measurement category (Table 1).

3.2. Anti-Cancer Activity of NcML on MCF-7 Cell Lines

MCF7 cell viability is impacted by NcML's concentration (Table 2), with MCF-7 cell viability declining as NcML concentration rises. The findings also demonstrate that there is no discernible difference

Table 1. Particle size of ML and NcML

Characterization	ML (nm)	NcML (nm)
Particle Size D10	784.4	222
Particle Size D50	942.9	267.3
Particle Size D90	1136	323.2
Particle Size Mean by number	923	262
Particle Size Mean by intensity	942	268.9
Particle Size Mean by volume	955.9	271.5
Zeta Potential		-52.8

D10 means 10% from measured of sample; D50 means 50% from measured of sample; 90% means 90% from measured of sample

Table 2. Cytotoxicity assay of NcML to MCF-7

Concentration (mg/mL)	Cell viability (%)
0.007	99.43
0.015	97.45
0.031	97.05
0.062	95.26
0.125	95.24
0.25	93.81
0.5	91.32
1	88.07

in the rise in NcML's cytotoxicity against MCF7 between concentrations; for example, MCF-7 cell viability was 91.32% at 0.5 mg/mL and 88.07% at 1 mg/mL, indicating a mere 3.25% difference between the two.

Treatment of MCF-7 cells with NcML at 0.25, 0.5, 1, 2, and 4 mg/mL induced cellular senescence (Figure 1B–F). Senescence-associated β -galactosidase (SA- β -gal) staining identified senescent cells as green, a widely used biomarker of senescence. Qualitatively, the frequency of SA- β -gal-positive cells increased with dose (Figure 1), and the quantification is presented in Figure 2.

The percentage of MCF-7 cells undergoing senescence at concentrations of 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, and 4 mg/mL (Figure 2) showed results of more than 50% and a concentration-dependent manner, meaning the increase in the number of MCF-7 cells undergoing senescence depended on the NcML concentration. At the lowest concentration, 0.25 mg/mL, the number of MCF-7 cells undergoing senescence was 56.22%, and at the highest concentration, 4 mg/mL, the number of MCF-7 cells undergoing senescence reached 81.92%.

Figure 3 shows that NcML induces cell-cycle arrest at the G2/M phase, evidenced by a significant increase in the proportion of cells in G2/M at 1 and 2 mg/mL compared with the vehicle control (0 mg/mL) on propidium iodide (PI) DNA-content flow cytometry. This pattern is consistent with activation of the G2/M checkpoint,

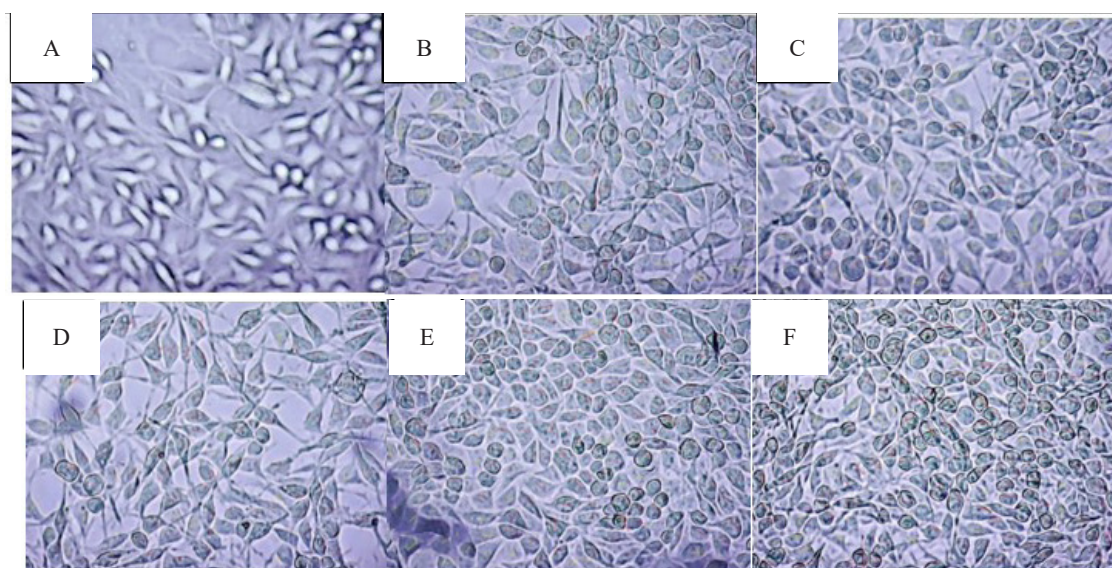


Figure 1. Senescence cell of MCF-7 treated by NcML which (A) negative control, (B) 0.25 mg/mL NcML, (C) 0.5 mg/mL NcML, (D) 1 mg/mL NcML, (E) 2 mg/mL NcML, (F) 4 mg/mL NcML (B-F cells undergo senescence).

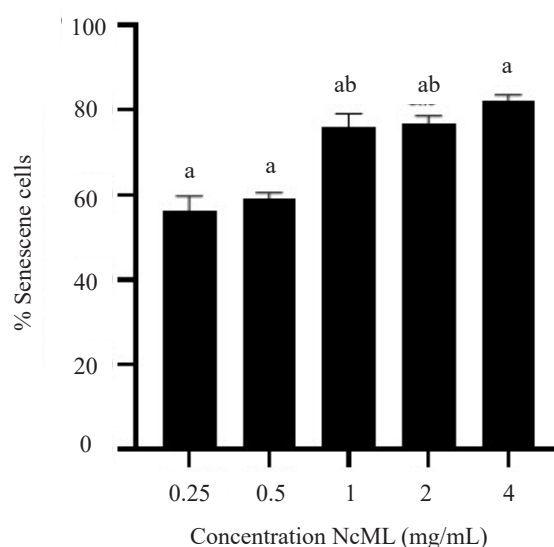


Figure 2. Persentation of senescence cells MCF-7. The data are presented in mean $\pm$ standard deviation. Data are presented as mean $\pm$ SD. Different symbols (a, ab, b) display a significant difference ($p < 0.05$) among treatments. ANOVA were used for data interpretation ($n = 3$).

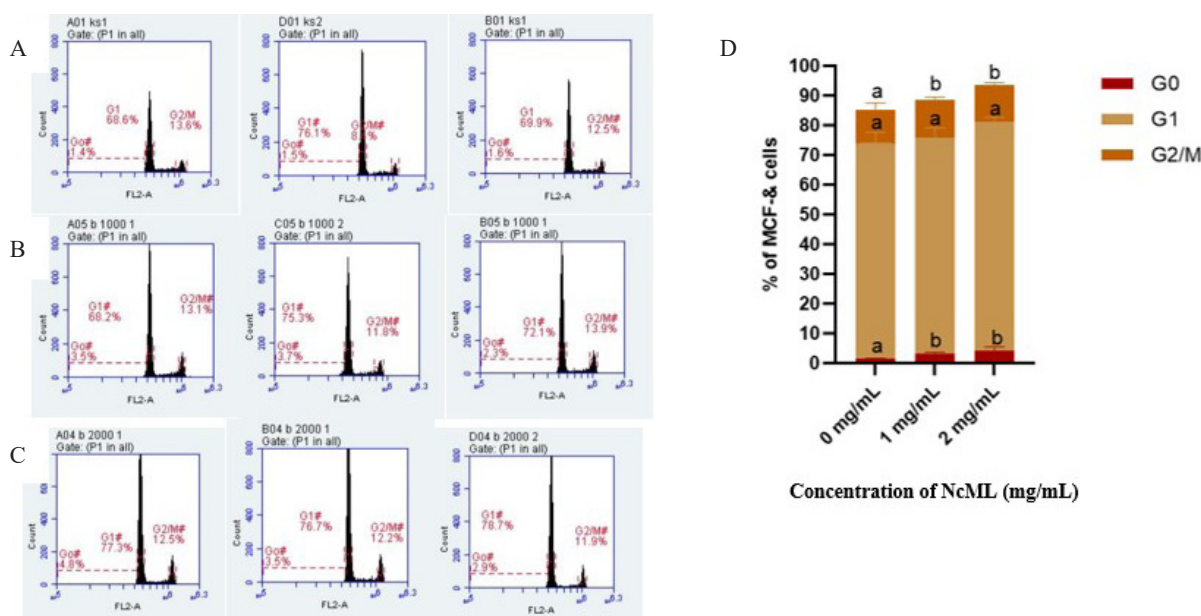


Figure 3. Distribution of Cells Cycle MCF-7 (A) results of cell cycle MCF-7 treatment with 0 mg/mL NcML, (B) results of cell cycle MCF-7 treatment with 1 mg/mL NcML, (C) results of cell cycle MCF-7 treatment with 2 mg/mL NcML, (D) distribution cell cycle of MCF-7 treatment by NcML. Data are presented as mean $\pm$ SD. Different symbols (a, b) display a significant difference ($p < 0.05$) among treatments. ANOVA were used for data interpretation ($n = 3$)

potentially reflecting engagement of the DNA-damage response in NcML-treated cells.

3.3. Anti-Inflammation Activity of NcML

The antioxidant activity against DPPH inhibition (Table 3) from a concentration of 0.25 mg/mL to 4 mg/

mL shows an increase. This indicates that the higher the concentration of NcML, the higher its antioxidant activity or the inhibition activity of NcML against DPPH free radicals in a concentration-dependent manner, meaning it depends on the concentration used. The lowest antioxidant activity of NcML is at a concentration of 0.25 mg/mL

with an activity of 34.19%, while the highest antioxidant activity, which is 43.68%, is at a concentration of 4 mg/mL. The percentage values of DPPH inhibition activity obtained from concentrations of 0.25 mg/mL to 4 mg/mL are 34.19; 35.81; 39.26; 40.81; 43.68.

At concentrations of 0.25 mg/mL and 0.5 mg/mL, sodium diclofenac does not show any inhibitory effect on BSA denaturation (Figure 4), unlike NcML, which already exhibits BSA denaturation inhibition at a concentration of 0.25 mg/mL by 5.57% and at a concentration of 0.5 mg/mL by 82.07%. Additionally, it can be seen (Figure 4) that at a concentration of 1 mg/mL, NcML also shows an inhibition activity value of 85.65%, which is higher than sodium diclofenac at the same concentration, which is only 76.49%.

4. Discussion

The size of NcML, which falls within the range of 222-323.2 nm, already meets the standard to be referred

to as a nanocrystal sample, even though generally something is considered to fall into the nano category if it has a size <100 nm. This definition is indeed officially adopted by the Food & Drug Administration (FDA) of the United States, the European Commission (EC), and the European Medicines Agency, but according to the scientific community, the category of nanocrystals falls within the range of 100-1000 nm, so the size of NcML still falls into the category of nanocrystals (Castro 2021). The bioavailability of a material will improve as its size is below 100 nm, but at that size, a material also becomes increasingly unstable. Therefore, the ideal size for a material's bioviability and stability is in the range of 200-500 nm (Peltonen & Hirvonen 2010; Chogale *et al.* 2016). As written by Zhang *et al.* (2020), smaller sizes in drugs provide a larger surface area, which increases absorbance activity and has the potential to reduce the dosage used.

Besides particle size, the measurement of zeta potential value is also performed using the same method, namely DLS or Dynamic Light Scattering. NcML has a zeta potential value of -52.8 mV with an electrophoretic mobility of $-4.08 \times 10^{-5} \text{ cm}^2/\text{Vs}$. This value proves that NcML has very good stability because it is above the threshold value for stability, which is >30 mV. The zeta potential value of NcML being above this standard threshold indicates that NcML has good colloidal stability, thus the likelihood of aggregation or sedimentation is very small. This is due to the strong electrophoretic repulsion forces between particles, which are caused by the increased surface area value (Honary & Zahir

Table 3. Anti-inflammation activity of NcML

Concentration of NcML	DPPH inhibition	Denaturation protein inhibition
0.25 mg/mL	34.19±0.006 ^a	5.57±0.005 ^a
0.5 mg/mL	35.81±0.002 ^a	82.07±0.003 ^b
1 mg/mL	39.26±0.021 ^a	85.65±0.007 ^b
2 mg/mL	40.81±0.010 ^a	86.05±0.006 ^b
4 mg/mL	43.68±0.009 ^a	85.65±0.005 ^b

(Different symbols (a, b) display a significant difference ($p < 0.05$) among treatments. ANOVA were used for data interpretation ($n=3$))

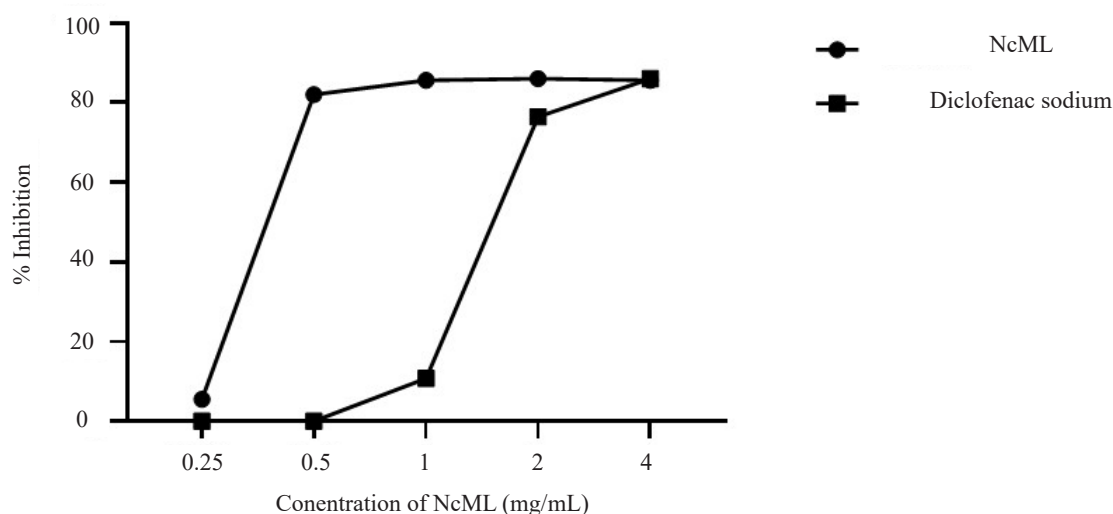


Figure 4. Inhibition activity of NcML and Diclofenac Sodium

2013; Zhang *et al.* 2020). These values are also known to have high levels of opsonization and internalization toward cells (Serda *et al.* 2022).

The cytotoxicity results show a concentration-dependent manner, meaning the effect of NcML on MCF7 viability is influenced by its concentration, with MCF-7 cell viability decreasing as the NcML concentration increases. The results also indicate that the increase in NcML cytotoxicity activity against MCF 7 from one concentration to the next does not differ significantly, such as at a concentration of 0.5 mg/mL with MCF 7 cell viability at 91.32% and at a concentration of 1 mg/mL, MCF-7 cell viability at 88.07%, showing only a 3.25% difference between the two. The cytotoxicity activity of NcML against MCF cells can be influenced by the size and zeta potential value of NcML, as it has a size >200 nm with a negative zeta value, making it difficult for NcML to penetrate the cell membrane because the cell membrane is also negatively charged (Honary & Zahir 2013; Zhang *et al.* 2020). Nevertheless, NcML still shows a toxic effect on MCF-7 cells. This cytotoxicity may be caused by eugenol and ethyl ester of hexadecanoic acid present in moringa leaves, which exhibit cytotoxic effects by altering membrane integrity and signaling pathways (Al-Asmari *et al.* 2015). Even compared to the toxic effects of moringa leaf extract alone in a study by Priyandoko *et al.* (2025) at a concentration of 0.05 mg/mL, which showed 100% viability in MCF-7 cells, NcML exhibited 91.32% viability at the same concentration in MCF-7 cells.

The senescence results of MCF-7 and NcML showed a better effect. Besides being able to induce senescence in over 50% of MCF-7 cells at a concentration of 0.25%, the difference between the concentrations of 0.25 mg/mL and 4 mg/mL was 25.7%, and statistically, they were significantly different with a p-value <0.05. However, other NcML treatments did not show significant differences from each other, such as the 0.25 mg/mL concentration, which caused senescence of 56.22%, not significantly different from the 0.5, 1, and 2 mg/mL concentrations, which caused senescence in MCF-7 cells of 59.16%, 76.05%, and 76.81%, respectively. The anti-cancer activity of NcML, which induces senescence, is similar to the effects of radiotherapy and the drug doxorubicin, as shown in the study of George *et al.* (2024).

Senescence is a cellular response that occurs when there is prolonged cell cycle arrest due to extracellular and intracellular stress (George *et al.* 2024; Tóth *et al.* 2024). In addition to the biomarker β -galactosidase used

in this study, senescent cells can also be identified by several disrupted metabolic pathways, including purine metabolism, D-glutamine and G-glutamate metabolism, disrupted biosynthesis of several non-essential amino acids such as arginine, proline, and cysteine, as well as disrupted metabolism or degradation processes in essential amino acid metabolic pathways such as methionine, leucine, valine, and threonine, in lipid metabolism and in energy metabolism (George *et al.* 2024). In the study by George *et al.* (2024), it is also stated that senescence in MCF-7 breast cancer cells is indicated by the presence of damaged double-strand DNA through YH2X staining and the increased levels of p12 and p16.

NcML can also be seen to inhibit the cell cycle at the G2/M phase due to a significant increase in the percentage of cells from the NcML treatment groups of 1 mg/mL and 2 mg/mL, as analyzed by flow cytometry using PI staining in the G2/M phase compared to the control group, or 0 mg/mL. This indicates an increase in the percentage of cells with membrane and DNA damage, causing these cells to be held back by the cell cycle checkpoint mechanisms. As stated by Han *et al.* (2013), in the data of cell distribution by flow cytometry with PI staining, the increase in the percentage of cells in the stop or checkpoint phases such as G1, S, and G2/M indicates a significant halt in those phases. The arrest of the cell cycle at the G2/M phase indicates that these cells are held back by the checkpoint mechanisms in the cell cycle, preventing damaged or unfit cells from proceeding to mitosis or duplication (Liu *et al.* 2024; Sun *et al.* 2021). This can also serve as validation that NcML inhibits cancer development through its induction of senescence because the senescence mediators p21 and p16INK4 also play an important role in maintaining cell cycle arrest. This cell cycle is related to cell proliferation and growth, which consists of gap phases (G1 and G2), synthesis phase (S), and mitosis or meiosis phase (M) (Øvrebø *et al.* 2022).

However, senescence, besides playing a role in inhibiting cancer, can also support cancer growth because continuous senescence can lead to chronic inflammation (Yamagata *et al.* 2024). Cells undergoing senescence will secrete pro-inflammatory cytokines, and if this continues, more pro-inflammatory cytokines will be secreted, which can harm normal cells and cause tissue dysfunction (Saleh *et al.* 2024; Yamagata *et al.* 2024). This can be overcome by the anti-inflammatory potential of NcML, as demonstrated by the results of DPPH inhibition activity and protein denaturation.

From the DPPH inhibition activity and BSA denaturation inhibition, it can be seen that the anti-inflammatory potential of NcML can even compete with and is better than NSAIDs because the BSA denaturation inhibition test can be used as one representation of tissues experiencing damage due to inflammation (Osman *et al.* 2016). The ability of NcML to suppress the denaturation process occurring in BSA is due to the antioxidant compounds present in NcML, which specifically bind to parts of BSA and protect it from damage caused by heating during the denaturation test (Williams *et al.* 2008). In addition to the 7 antioxidant compounds mentioned earlier, the content of vitamin C, vitamin E, kaempferol, and quercetin found in moringa leaves also plays a role as antioxidants (Sreelatha *et al.* 2011; El-sherbiny *et al.* 2024). These compounds that act as antioxidants have the potential to reduce degenerative diseases related to oxidative damage, such as cancer and cardiovascular disorders (Segwatibe *et al.* 2023).

Therefore, in addition to its anti-cancer activity, NcML also has anti-inflammatory activity that protects normal cells surrounding cancer cells that have undergone senescence due to NcML's anti-cancer mechanism, which induces senescence and can lead to an increase in ROS in normal cells. Abedi Tameh *et al.* (2024) clarified this by stating that while antioxidants in healthy cells protect the cellular metabolic balance by limiting ROS levels, these secondary metabolites have the opposite effect in cancer cells, acting as pro-oxidants and raising ROS levels.

Acknowledgements

Thank you to the Kemdiktisaintek and Universitas Pendidikan Indonesia for placing their trust in us by funding this research. The authors would like to express sincere gratitude to all parties who assisted in the smooth progress of this research series, including PT FAST, PT NIG, PPNN Institut Teknologi Bandung, the Central Laboratory, and the Pharmaceutical Laboratory of Universitas Padjajaran.

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