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RESEARCH ARTICLE

Cytotoxic Activity of Ethanolic Extract of *Zanthoxylum acanthopodium* DC. Fruit as Phytosomal System against MCF-7 Cell Line

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Abstract

Zanthoxylum acanthopodium DC., known as andaliman, contains bioactive compounds with potential anticancer properties. This study investigated the cytotoxic activity of the ethanolic extract of *Z. acanthopodium* and its phytosomal formulation (Phytosome-EEA) against the MCF-7 breast cancer cell line. Phytosomes were prepared using antisolvent precipitation and optimized based on phospholipid ratios, reflux temperature, and n-hexane droplet rate. The phytosomes were characterized by particle size, polydispersity index, zeta potential, entrapment efficiency, stability tests, and morphology study by Transmittion Electron Microscopy (TEM). WST-1 assay at 450 nm was used to assess cytotoxicity against HEK 293A and MCF-7 cells, while apoptosis was evaluated using Annexin V-PI staining. The optimized phytosome formulation yielded nanoparticles with an average size of 192.4 nm, polydispersity index of 0.783, a zeta potential of −36.7 V, and an entrapment efficiency of 90.26%. When stored at 4, 25 and 40 °C for 14 days, there were no significant changes in particle size, zeta potential, or cytotoxicity, indicating high physical stability. Both the ethanolic extract and phytosomes exhibited cytotoxic activity against MCF-7 and HEK 293A cells, with IC_{50} values of 100.2 and 109.2 µg/mL, respectively. The cell death induced by EEA occurred primarily via apoptosis, while Phytosome-EEA promoted increased late apoptosis. Although the selectivity index remained < 1, the phytosomal formulation maintained consistent cytotoxic activity and improved physicochemical characteristics compared to the crude extract.

Keywords: *Zanthoxylum acanthopodium*, Phytosomes, Cytotoxic, Apoptosis, MCF-7 cell

Introduction

Zanthoxylum acanthopodium DC., commonly known as andaliman, has been identified as a promising candidate for anticancer drug development due to its content of flavonoids, alkaloids, and tannins—compounds known to induce apoptosis and inhibit cancer cell proliferation [1,2]. Prior studies have reported cytotoxic activity of *Z. acanthopodium* extract against MCF-7 and T47D breast cancer cell lines, while showing low toxicity toward normal cells [3].

Most cases of cancer is breast cancer with an incidence rate of 19.18% [4]. Anticancer agents ideally have selective toxicity with ability to destroy cancer cells without damaging normal tissue cells. Currently, cancer treatment in Indonesia uses chemotherapy such as cyclophosphamide, methotrexate, and doxorubicin.

Chemotherapy and radiation agents are extremely toxic and may harm adjacent healthy cells. The primary adverse effects of chemotherapy include nausea, vomiting, diarrhea, mucositis, alopecia, and constipation, among others. These side effects significantly impact the quality of life of cancer patients [5,6].

MCF-7 is a prevalent breast cancer cell line, which has been used for many years. MCF-7 is a suitable line cell model for breast cancer investigations worldwide. Over time, MCF-7 has produced more data than any other breast cancer cell line. MCF-7 is an estrogen receptor (ER)-positive and progesterone receptor (PR)-positive cell line exhibiting anti-hormonal properties. They also show less aggressive and less invasive activity compared with other cell lines [7] .

The development of anticancer agents from plant has been growing in the late 1950s [8]. *Z. acanthopodium* DC. or known as andaliman, easily be found in North Sumatra, Indonesia and had been widely used as traditional cooking spice [9]. Phytochemical Screening results of 70% ethanol extract of its fruit showed that it contained flavonoids, saponins, tannins, steroids and coumarins which is known to have anticancer activity [10.] The 70% ethanolic extract exhibited an IC_{50} value of 84.1 + 0.47 µg/mL in the antioxidant activity assay, which was significantly higher than that of n-hexane and ethyl acetate. Previous studies have also supported the anticancer potential of *Zanthoxylum acanthopodium*. Arsita *et al.* [3] reported that the ethanolic extract of *Z. acanthopodium* seeds showed cytotoxic activity against MCF-7 breast cancer cells with an IC_{50} value of 221.31 µg/mL, indicating its potential as an anticancer agent through apoptosis induction mechanisms. The inhibition activity on the proliferation of MCF-7 cells showed selectivity index value of 6.08 against cervical cancer cells and Vero cells [11]. The extract is considered active when the IC50 value is from 10 to 100 µg/mL and notably active when the IC50 value is between 100 and 500 µg/mL [12]. The extract is classified as selective if the Selectivity Index value > 3 [13].

Secondary metabolites from plant such as flavonoids, glycosides and phenols are hydrophilic compounds, soluble in polar solvents, affecting their ability to interact with lipid-based compounds, resulting in poor bioavailability, and inhibiting compounds from crossing the lipid-rich outer membrane [14]. Phytosome technology offers a strategy to overcome these barriers by forming molecular complexes between phospholipids and hydrophilic plant compounds, thereby enhancing membrane permeability, stability, and systemic absorption [15,16]. Despite the established benefits of phytosomes for other herbal actives, there has been limited investigation into their application with *Z. acanthopodium* [14]. Therefore, development of phytosomal system of *Z. acanthopodium* ethanolic extract and evaluation on its activity against MCF-7 cell lines as well as its selectivity against normal HEK 293-A cells had been conducted.

Materials and methods

Materials

This study utilized *Zanthoxylum acanthopodium* fruit, verified by plant identification performed by Achmad Mabrur, Skm, M.Kes. at the UPT Herbal Materia Medica Laboratory in Batu, Malang, East Java, in 2023, with Submission No. 067/ 077/ 102.20/ 2023. The chemicals used were ethanol 70%, soya lecithin, dichloromethane, n-hexane, silica gel GF254 (Merck), chloroform, methanol, ammonia, Dragendorff's reagent, phosphotungstic acid, dimethyl sulfoxide (DMSO) (Sigma), HEK cells, MCF-7 cell line, growth media (DMEM), WST-1 (Water Solubel Tetrazolium-1) (Sigma), phosphate buffer saline (PBS), and Trypsin.

Method

Digestion maceration

Dried *Z. acanthopodium* fruit powder (400g) was extracted by 3 L ethanol 70%, for 3×24 h with occasionally stirring. Filtrate was evaporated by vacuum rotary evaporator at 50 °C, 75 rpm until concentrated. Furthermore, evaporation was carried out using a water bath. The extract (EE) was stored at 4 °C

Phytochemical screening

Phytochemical screening of ethanol extract of (EET) included the examination of alkaloid, phenolic, flavonoid, quinone, monoterpene, and steroid/triterpenoid compounds.

Phytosome preparation

Phytosomes were synthesized via the antisolvent precipitation technique. Specific amounts of extract and phospholipids (ratio 1:1 and 1:2), were added into a 100 mL round bottom flask. The mixture was refluxed using 20 mL of dichloromethanes at 40, 50, or 60 °C for 3 h, then was concentrated to 5 - 10 mL. Subsequently, 20 mL of n-hexane was added with continuous stirring to precipitate the phytosome, which was subsequently filtered and collected. The phytosome powder was preserved in a desiccator overnight. The produced phytosome powder was contained in colored glass vials and maintained at ambient temperature [17].

Characterization of phytosome

Particle size, polydispersity index (PDI) and zeta potential

The particle size, PDI, and zeta potential of phytosomes were assessed using a Particle Size Analyzer (PSA) employing the dynamic light scattering (DLS) technique. Measurements were conducted utilizing a Horiba Scientific SZ-100, with a tenfold dilution of the material in aqueous solution at ambient temperature [18].

Entrapment efficiency (EE)

The ultracentrifugation method was used to determine the sorption efficiency of the phytosomes. The formulation solution was centrifuged at 15,000 rpm at 4 ºC for half an hour. The supernatant was obtained, and the quantity of the free active ingredient was quantified by UV-Vis spectrophotometry at 360 nm (Shimadzu-1601, Japan). The active substance of the phytosome was measured as quercetin as the flavonoid which is the major component of the extract. The EE was calculated as below [19]:

$$\text{Entrapment efficiency} = \frac{\text{total drug} - \text{free drug}}{\text{Total drug}} \times 100 \quad (1)$$

Stability test

The stability test was carried out at 4, 25 and 40 °C. Samples were stored in glass bottles and placed in a refrigerator (4°C) and then in a desiccator (25°C) and climatic chamber (40°C). Particle size, PDI, and zeta potential were assessed from day 0 to day 4 [20].

Morphology observation using transmission electron microscopy (TEM)

The morphology of the phytosomes was examined utilizing a transmission electron microscope (JEOL JEL-1400). Samples were diluted with distilled water at a ratio of 1:20. A droplet of phytosome solution was deposited onto a carbon-coated copper grid. A 2% uranyl acetate solution was subsequently used to adversely stain the material. The material was let to air dry at ambient temperature for 15 min prior to imaging. The phytosomes were seen using transmission electron microscopy (TEM) at an accelerating voltage of 100 kV [21,22].

Cytotoxicity test by WST-1 method

Preparation of test materials

The extract (EE) was dissolved in dimethyl sulfoxide (DMSO), and mixed by vortex until completely dissolved. Next, it was diluted by culture media to obtained concentration of 500, 250, 125, 62.5, and 31.25 µg/mL [13].

HEK cell/MCF-7 cell assay

HEK cells and MCF-7 cells were cultivated in 96-well microplates to a density of 1×10^4 cells per well and incubated for 24 h. Before the insertion of the sample solution, the media was changed with fresh material. Following the incorporation of sample solutions at varying concentrations with DMSO as a cosolvent, they were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Upon completion of incubation, the cells were rinsed, after which 100 µL of culture media and 10 µL of WST-1 at a concentration of 5 mg/mL were added. To assess viability, the cells were cultured for 4 h in a 5% CO_2 incubator at 37 °C, and the results were measured using an ELISA reader at a wavelength of 450 nm. Percent live cells were calculated using the formula:

$$\% \text{ viability} = \frac{\text{Absorbance of treated cells - Absorbance of media}}{\text{Cell control absorbance - Media control absorbance}} \times 100\% \quad (2)$$

Cytotoxic activity was expressed as IC50 which was analyzed by regression analysis using Graphpad prism application. The Selectivity Index is calculated using the equation below [13]:

$$\text{Selectivity Index} = \frac{IC_{50}\ \text{HEK Cells}}{IC_{50}\ \text{MCF-7 Cells}} \quad (3)$$

Apoptosis assay

MCF-7 cells (5×10^5 cells/well) were grown in a 6-well microplate and incubated for 24 h. The samples were subsequently introduced into the well and incubated for 24 h. Subsequent to incubation, the media from each well were gathered in a 15 mL conical tube, rinsed with PBS, and 250 µL of Trypsin was introduced, followed by a 3-minute incubation, after which 1 mL of culture medium was added. The samples were centrifuged at 6,000 rpm for 5 min, after which the supernatant was discarded. Subsequent to the addition of 1 mL of PBS, the medium was transferred to a conical tube and centrifuged at 2,000 rpm for 3 min, following which the supernatant was discarded. Subsequent to the incorporation of Anexin, the sample was analyzed using a flow cytometer [13].

Results and discussion

Phytochemical screening

Phytochemical analysis revealed that the ethanol extract of *Z. acanthopodium* fruit contains alkaloids, polyphenols, flavonoids, monoterpenes, and steroids. This is due to the ethanol solvents which is very effective in extracting fixed oils, fats, waxes, alkaloids, flavonoids, polyphenols, tannins, saponins, aglycones and glycosides [23]. The ethanolic extract of *Zanthoxylum acanthopodium* DC. (andaliman fruit) used in this study was standardized prior to formulation to ensure reproducibility and quality. The extract presented as a brown, viscous liquid with a strong odor. It demonstrated a moisture content of 26.21%, total ash value of 3.12 ± 0.08%, and acid-insoluble ash of 0.92 ± 0.08%. Microbial analysis indicated total plate count of 19,447 CFU/mL and yeast/mold count of 4.11×10^2 CFU/mL, both within pharmacopeial limits. Phytochemical quantification revealed a total flavonoid content of 3.70 mgQE/g and phenolic content of 37.98 mgGAE/g. The extract had a specific gravity of 0.81 g/mL. These parameters ensured that the extract complied with quality control requirements for further formulation.

Phytosome production

The phytosome production used the antisolvent precipitation method with variations in phospholipid: Extract ratio, n-hexane droplet speed and reflux temperature variations. The parameters to be determined in the optimization process were particle size, polydispersity index (PDI), and zeta potential. The assessment of particle size is a critical parameter as it influences drug loading, drug release, and the stability of nanoparticles. The PDI value of the particle size distribution serves as a criterion for size homogeneity [24].

Zeta potential is an electrical potential associated with particle mobility, frequently employed to evaluate the surface charge characteristics of nanoparticles. This is important for evaluating physical stability, determining the effectiveness of surface coatings or drug adsorption on nanoparticles [25]. Zeta potential indicates the stability of phytosomes in a medium, since charged particles generate repulsive forces to uphold stability [26]. The particle size, PDI and zeta potential values of the phytosomes can be seen in **Table 1**.

Table 1 Particle size, PDI and zeta potential of *Z. acanthopodium* extract phytosomes.

Formula	Phospholipid: extract ratio	Drip speed of n-hexane	Reflux temperature (°C)	Particle size (nm)	PDI	Zeta potential (mV)
1	1:1	4 mL/min	50 °C	192.4 ± 23.2	0.783 ± 0.155	−33.2 ± 3.2
2	1:1	2 mL/min	50 °C	367.4 ± 37.5	0.429 ± 0.126	−36.7 ± 2.8
3	1:1	6 mL/min	50 °C	400.2 ± 41.6	0.714 ± 0.143	−28.7 ± 3.5

The characterization on the phytosomes showed that Formula 1 revealed particle size as much as 192.4 nanometer with optimum zeta potential −33.2 and PDI of 0.783. The PDI value of the optimized phytosome was 0.783, which indicates a broad size distribution. Although this exceeds the commonly accepted

pharmaceutical threshold of PDI < 0.5 for uniform nanosystems, it may be attributed to the complex composition of the plant extract. The zeta potential of –33.2 mV suggests good electrostatic stability. Data presented are based on triplicate measurements (n = 3). Overall, while the system demonstrates preliminary stability, further refinement is needed to improve homogeneity and reduce batch-to-batch variability.

Phytosomes are biodegradable, spherical delivery systems with a diameter of 50 - 500 nm, forming complexes between phospholipids and active plant compounds through the formation of chemical bonds that make them more stable [27]. The polydispersity index value is close to 1, which indicates a homogeneous particle size distribution [24]. Phytosome emulsions with zeta potential greater than or less than 30 mV are said to be stable [26]. A study on the optimum phospholipid-to-extract ratio showed that higher extract ratio (F5) resulted in a larger particle size due to increased viscosity. A higher phospholipid ratio (F4) also led to an increase in particle size, although the size was still smaller compared to that of F5 (**Table 2**). Further study showed that reflux temperature did not affect the particle size of the phytosome (data not shown).

Table 2 Particle size, PDI and zeta potential of *Z. acanthopodium* extract phytosomes with different ratio of extract to phospholipid.

No	Phospholipid: extract ratio	Drip speed of n-hexane	Reflux temperature (°C)	Particle size (nm)	PDI	Zeta potential (mV)
1	1:1	4 mL/min	50 °C	192.4	0.783	−33.2
4	2:1	4 mL/min	50 °C	380	0.675	−35.2
5	1:2	4 mL/min	50 °C	1367. 9	1.059	−31.6

The selection of phytosome as the delivery system for the ethanolic extract of *Zanthoxylum acanthopodium* was based on its superior ability to enhance the bioavailability of phytoconstituents, especially those that are hydrophilic, poorly lipid-soluble, and exhibit limited cell membrane permeability, such as flavonoids, alkaloids, and tannins - major constituents identified in our extract [28].

Unlike conventional delivery systems such as simple liposomes or polymeric nanoparticles, phytosomes form a molecular complex between phospholipids and phytoconstituents, resulting in improved absorption, stability, and cellular uptake [29,30]. This complex formation involves hydrogen bonding and interaction of polar functional groups of the active constituents with the polar head of phospholipids (e.g., phosphatidylcholine), leading to better integration into the lipid bilayer of biological membranes [27].

Additionally, studies have shown that phytosomal systems improve pharmacokinetic parameters, such as *Cmax*, *Tmax*, and *AUC*, compared to both free extracts and conventional nanoparticles [31,32] Moreover, phytosomes offer better physical stability, as demonstrated by our own results, which showed stable particle size and zeta potential across varying storage conditions (4, 25 and 40 °C), making them ideal for pharmaceutical formulation [17]. Furthermore, phytosomes are biocompatible and biodegradable, making them a safer and more favorable option for herbal-based delivery [33,34].

In contrast, other systems like solid lipid nanoparticles (SLNs), polymeric nanoparticles, or nanoemulsions may involve higher production complexity, cost, and less compatibility with natural compounds, especially in the context of traditional herbal formulations [33,35,36]

Entrapment efficiency

Elevated EE is beneficial as it facilitates sufficient medication delivery to target cells and prolongs drug contact duration [37]. The results showed that the EE of phytosome of the extract was fairly high (90.26% ± 0.006). The entrapment efficiency depends on the type of drug and drug- polymer interaction [38].

Transmission electron microscopy

Visual imaging using transmission electron microscopy of the Phytosome-Ethanol Extract of Andaliman Fruit (EEA) formula showed spherically shaped particles in the nanometer size range as shown in **Figure 1**.

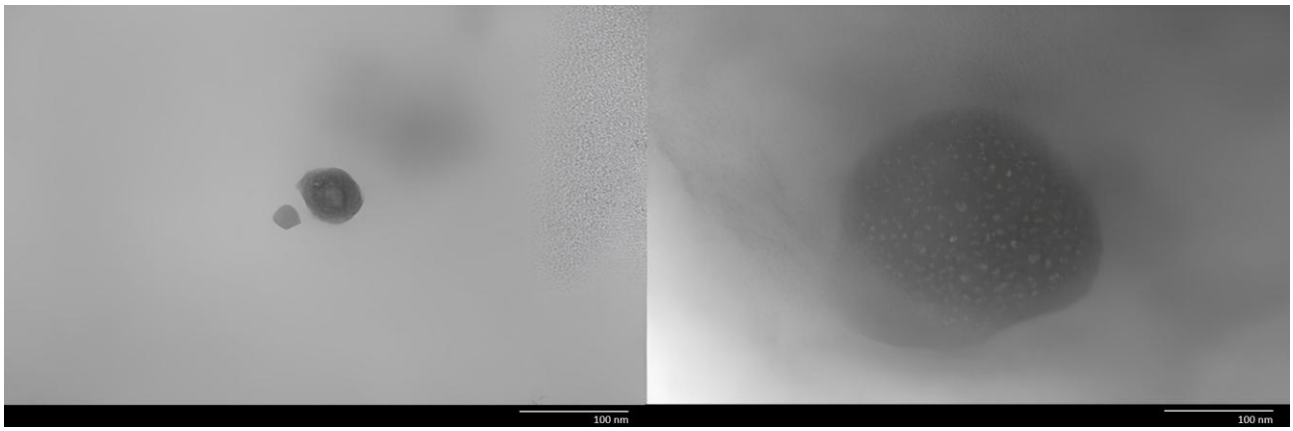

Figure 1 TEM micrograph of Phytosomes using a Transmission Electron Microscope.

Stability test

Phytosomal stability is another important factor in the successful design of delivery system formulations. Stability studies were conducted to explore the changes in phytochemicals of phytosomes during storage [39]. The physical stability of phytosome was observed at 4 °C (refrigerator), 25 °C (desiccator), and 40 °C (climatic chamber) in **Table 3**. The results indicated that the particle size, PDI and zeta potential after 2 weeks of storage did not change did not changed significantly, which indicates that these phytosomes are quite stable during storage

Table 3 Stability test results of phytosomes.

Stability Test	0 day			2 weeks		
	Particle size (nm)	PDI	Zeta potential (mV)	Particle size (nm)	PDI	Zeta potential (mV)
Refrigerator	192.4	0.783	−33.2	198.8	0.779	−29.2
Desicator	192.4	0.783	−33.2	193.8	0.840	−32.4
Climmatic chamber	192.4	0.783	−33.2	214.2	0.714	−33.4

Cytotoxic activity

Cytotoxic testing was performed to assess the potential toxicity of extracts and phytosomes on HEK 293-A cells and MCF-7 cells, quantified in terms of IC_{50} (Inhibitory Concentration) values, while their selectivity was represented by the selectivity index.

Cytotoxic test of the Extract against MCF-7 Cells was conducted by using the WST-1 Method. Water-soluble tetrazolium (WST) or tetrazolium salts have sulfonate groups that can be added to the phenyl ring to increase their solubility in water. WST-1 is impermeable to cells; hence the reduction transpires extracellularly via plasma membrane electron transfer [40]. Phenylazotype tetrazolium salts are easily reduced by nicotinamide adenine dinucleotide phosphate-oxidase (NADH) or other reducing agents to produce orange or purple formazan dyes that can be measured for absorbance with a micro plate reader at a wavelength of 450 nm.

The efficacy of the extract at concentrations of 7.8125, 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL

against HEK 293-A and MCF-7 cells was determined using the formula shown below:

$$\% \text{ viability} = \frac{\text{Absorbance of cells with treatment} - \text{Absorbance of media}}{\text{Absorbance of Cell control} - \text{Absorbance of media control}} \times 100\% \quad (4)$$

Percentage of survival from HEK 293-A cells and MCF-7 cells with extract treatment is presented in **Figure 2**

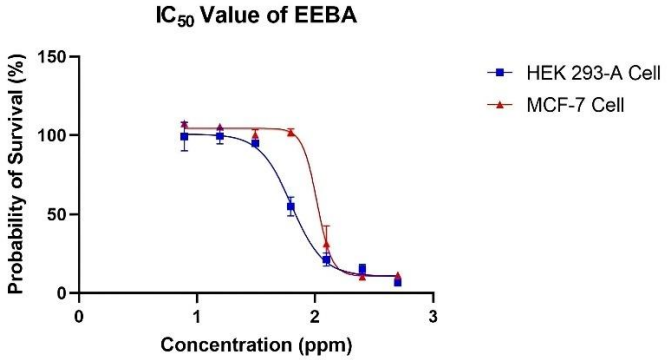

Figure 2 IC_{50} Determination of Ethanol Extract of *Z. acanthopodium* DC. Fruit against MCF-7 and HEK293-A Cells.

Curve of respond doses from HEK 293-A (normal cell) and MCF-7 (breast cancer cell) treated by various concentration of ethanolic extract of *Z. acanthopodium* DC were analized by GraphPad Prism software (**Figure 2**) showed that IC_{50} values of the extract against HEK 293-A cell was 67.42 ppm with 95% CI (Confidence Interval) as much as 59.91 to 76.40 ppm while that against, MCF-7 cell was 100.2 ppm with 95% CI as much as 89.90 to 120.77 ppm. The results showed that toxicity of the extract against normal cell was higher than that of cancer cell to which the IC_{50} of HEK 293-A cell was lower.

Based on toxicity classification, IC_{50} value in the range of 50 - 100 ppm is categorized as moderate while IC_{50} > 100 ppm is concluded as low toxicity. Based on the results, the ethanolic extract of *Z. acanthopodium* DC revealed moderate toxicity against HEK 293-A cell and low toxicity against MCF-7 cell. Selectivity Index (SI) value which is calculated as ratio of IC_{50} from normal cell against cancer cell was 0.67. This indicated that the extract was not selective enough against cancer cell since the ideal IS index for anticancer agent should > 3 [41]. Thus, the extract was less selective against MCF-7 cancer cells. This means that Phytosome-EEA is able to kill MCF-7 cancer cells but is also able to kill normal cells.

The cytotoxic effect is the result from the presence of secondary metabolites in the extract such as flavonoids, alkaloids, and tannins which have been proved to have the ability to induce apoptosis, suppress the cell cycle, or trigger oxidative stress [42,43]. At low extract concentrations (≤ 31.25 ppm), cytotoxic effects on both cell types were insignificant, which may be due to low concentration of the active compounds to give the activity. However, at high concentrations (≥ 62.5 ppm), the toxic effects become more pronounced, especially in HEK 293-A cells due to the greater sensitivity of normal cells to oxidative stress compared to cancer cells. This is relevant to research [44,45]. which showed that secondary metabolites such as alkaloids can increase reactive oxygen species (ROS) leading to DNA damage and apoptosis, especially at high concentrations. Low SI value of the extract and high concentration to give the activity indicated the need of further optimization, such as fractionation to separate more specific active compounds against cancer cells [46-48].

Observations conducted with an inverted microscope at a magnification of 10×10 revealed morphological differences between HEK 293-A cells and control MCF-7 cells, as well as between HEK 293-A cells and MCF-7 cells treated with extract (**Figure 3**). HEK 293-A cells and MCF-7 cells in the control group had a polygonal morphology with distinct cell nuclei. HEK 293-A cells and MCF-7 cells that succumbed to extract treatment exhibited a dispersed distribution with a darkened core, adhered to the well bottom, and resulted in reduced cell density relative to the control.

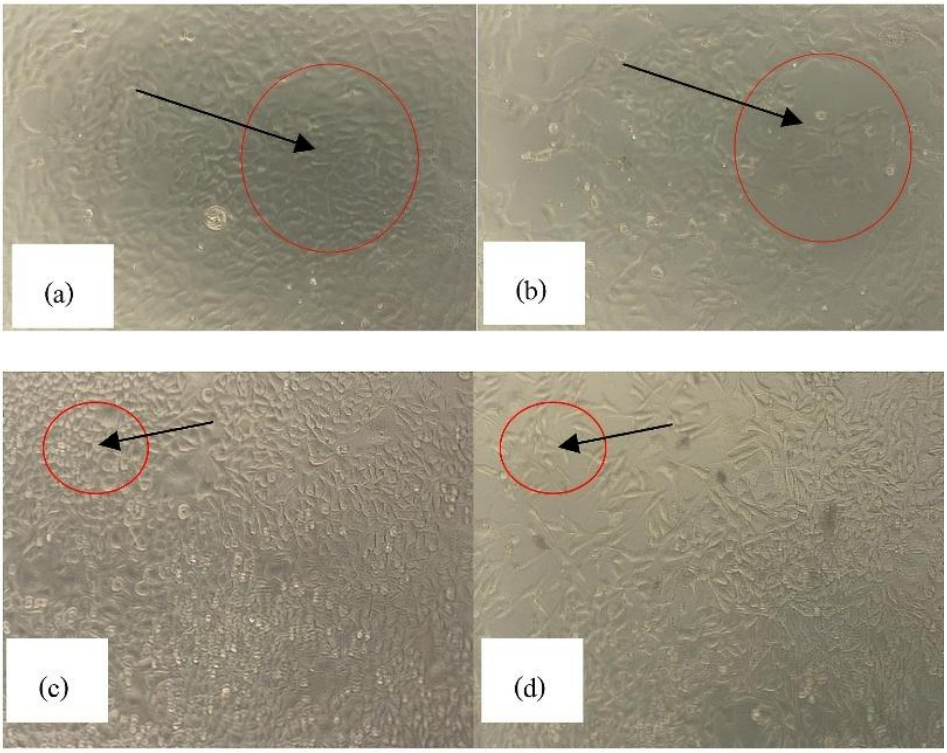

Figure 3 HEK 293-A cells (a) HEK 293-A cell control, (b) HEK 293-A cells after treated with 125 ppm extract (c) MCF-9 cells control, (d) MCF-7 cells after treated with 125 ppm Extract.

The phytochemical screening of the extract revealed the presence of alkaloids, flavonoids, tannins, and steroids/triterpenoids. The presence of these secondary metabolite compounds indicates the presence of bioactive compounds that function as anticancer. Alkaloids are very important compounds in plants of the genus Zanthoxylum where alkaloids have anticancer activity. The alkaloid group can cause damage and shrinkage of the cell membrane so that the components of the membrane will change and the physiological process of the membrane will be disrupted.

Flavonoids impede proliferation by obstructing oxidative mechanisms that may lead to cancer onset. This method is facilitated by a reduction in the enzymes xanthine oxidase, cyclooxygenase (COX), and lipoxygenase (LOX), which are essential for the peroxidation process, thereby postponing the cell cycle. Flavonoids exhibit anticancer action via inducing apoptosis. Flavonoids suppress the production of topoisomerase I and topoisomerase II enzymes, which are involved in catalyzing DNA rotation and relaxation. Inhibitors of topoisomerase enzymes will stabilize the topoisomerase complex, resulting in the cleavage and destruction of DNA. DNA damage can induce the production of pro-apoptotic proteins, including as Bax and Bak, while diminishing the expression of anti-apoptotic proteins, specifically Bcl-2 and Bcl-XL. Consequently, the proliferation of cancer cells is suppressed. Flavonoids will elevate p53 levels, hence enhancing apoptosis [43]. Tannin will elevate p27, resulting in cell cycle arrest and subsequent alterations in the permeability of the MCF-7 cell membrane. This is indicated by changes in cell morphology after administration of andaliman fruit extract to MCF-7 cell culture [43].

Curve of respond doses from HEK 293-A (normal cell) and MCF-7 (breast cancer cell) treated by Phytosome of the extract at concentration of 8.58 to 549.45 ppm showed that the IC_{50} values were 74.09 ppm against HEK 293-A with 95% CI as much as 65.44 - 84.48 ppm (**Figure 4**). IC_{50} value against MCF-7 was 109.2 ppm with 95% CI between 98.54 -129.7 ppm. It can be concluded that toxicity against normal cell was higher than that against cancer cell. Compared with IC_{50} from the extract, which was 67.42 ppm against HEK 293-A and 100.2 ppm against MCF-7, phytosome

formulation showed slight increase in IC50 value which assumed as lower toxicity. Nevertheless, Selectivity Index (SI) of phytosome was 0.68 which was not differ from SI value of the extract.

The Selectivity Index (SI) of both the extract and phytosomal formulation was < 1, indicating poor selectivity toward cancer cells. This could be attributed to the use of crude extract containing multiple compounds with non-specific cytotoxic effects. Contrary to expectations, the phytosome did not significantly enhance cytotoxic activity or selectivity. This may be due to passive diffusion mechanisms, lack of targeted delivery, or slower release kinetics inherent to the phospholipid bilayer system. In apoptosis analysis, the phytosome group showed low early apoptosis but elevated late apoptosis, suggesting a delayed apoptotic effect. These findings indicate that while phytosome improves formulation stability, further optimization—particularly involving targeted delivery and compound fractionation—is necessary to improve therapeutic specificity.

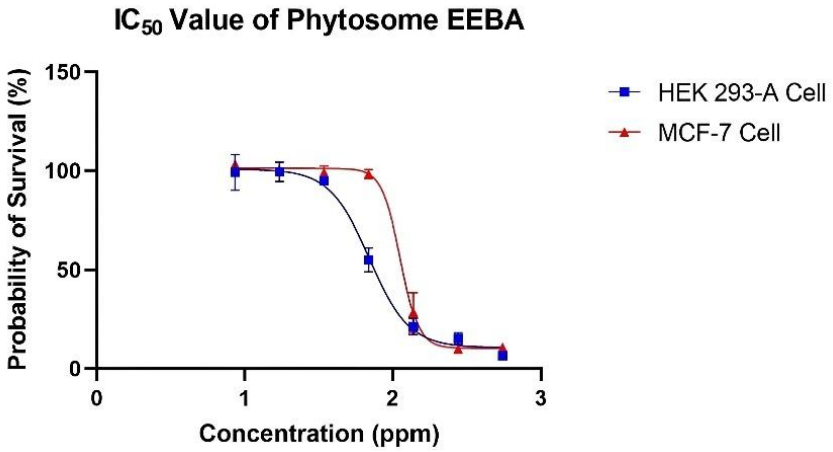

Figure 4 IC_{50} Determination of phytosome of ethanolic extract of *Z. acanthopodium* DC. on MCF-7 and HEK293-A Cells.

The higher toxicity value to normal cells compared to cancer cells in phytosome formulations is due to the ability of phytosomes to control the gradual release of active compounds. The lipid bilayer structure in the phytosomes protects the active compounds from direct release thereby slowing their distribution and reducing the initial overexposure to normal cells. In contrast to extracts without formulation that directly release active compounds to cells, phytosomes make active compounds more stable in the body and prevent unwanted interactions with normal cells [34,49,50]. In addition, phytosomes also help increase the availability of active compounds, especially those that are difficult to dissolve in water such as flavonoids and alkaloids, so that these compounds are more effective in achieving their targets [51-53]. Phytosomal lipid bilayers have a tendency to release active compounds in the area of cancer cells due to their acidic environment, so phytosomes are more effective at attacking cancer cells than normal cells [27,54,55]. However, the low Selectivity Index (SI) value indicates that the active compounds in this formulation are not yet specific enough for cancer cells. Therefore, further development is needed, such as adding molecules that can recognize cancer cells or modify the lipid bilayer structure to be more sensitive to the conditions surrounding cancer cells [56-58]. With optimization, phytosomes can become a safer and more effective anticancer drug delivery system.

Observational results utilizing the same microscope revealed a morphological distinction between HEK 293-A cells and the control. MCF-7 cells co-cultured with HEK 293-A cells and MCF-7 cells subjected to phytosome extract treatment. HEK 293-A cells and MCF-7 cells subjected to phytosome-extract treatment exhibited a tendency to disperse with a central black coloration, resulting in reduced cell density relative to the control group.

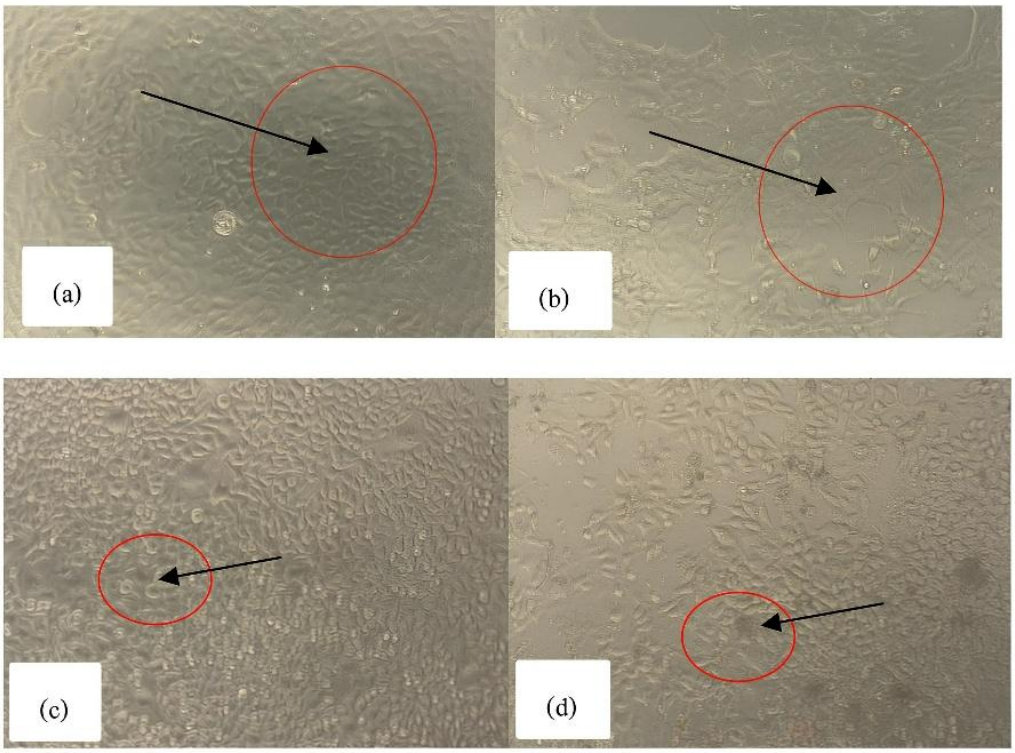

Figure 5 HEK 293-A cells (a) HEK 293-A cell control, (b) HEK 293-A cells after treated with 125 ppm phytosome extract, (c) MCF-9 cells control, (d) MCF-7 cells after treated with 125 ppm phytosome extract

Research and clinical studies have shown an increase in the bioavailability and absorption of herbal medicines administered in conjugation with phytosomes [31]. The main benefit is the drastically increased bioavailability of phytoconstituents due to their association with phospholipids resulting in increased phytoconstituent absorption and distribution. With increased bioavailability, the absorption of the extract constituents increases. As the absorption of the major phytoconstituents increases, the dosage requirements decrease

Phospholipids are able to form chemical bonds with flavonoid molecular derivatives. The molecular docking model occurs due to the interaction of the phospholipid 20(S)-protopanaxadiol (PPD) complex. The hydrophobic region of the PPD framework is bordered by two hydrophobic extensions of the phospholipid molecule, whereas hydrogen bonds with the phospholipid backbone of the P = O segment are formed by one of the hydrophilic OH groups. Hydrogen interactions are the primary interactions within the phytosome vesicle system [59]. The use of phospholipids (lecithin), with the main component of phosphatidylcholine, in the process of forming vesicular phytosomes, in addition to being a carrier, its use can induce proliferation, provide mitogenic effects, and play a role in mediating cell membrane signaling, which can be one of the reasons for the limitations in this study.

Apoptosis tests of the extract and phytosome-extract

Apoptosis was assessed by flow cytometry. This technique facilitates the rapid enumeration of viable cells, necrotic cells, and apoptotic cells. This test employs Annexin V, a protein that selectively binds to phosphatidylserine present in the cell plasma membrane during apoptosis. Propidium iodide stains DNA in injured cells, whether necrotic or apoptotic, resulting in orange to red fluorescence. As cells traverse a laser beam, they become energized and emit scattered light, resulting in fluorescence [60,61]. The outcomes of apoptosis assessment using flow cytometry on MCF-7 cells following the administration of the extract and Phytosome-extract are presented in **Table 4** and **Figure 6**.

Table 4 Fluorescence average percentage of control, extract and phytosome-extract against MCF-7 cells.

Quadran	Treatment			
	Cell control	**Solvent control**	**Extract**	**Phytosome**
Viabel	95.50	90.10	86.80	89.23
Early apoptosis	0.92	1.03	6.56	1.23
Late apoptosis	3.32	7.73	6.47	8.12
Nekrosis	0.27	1.18	0.17	1.42

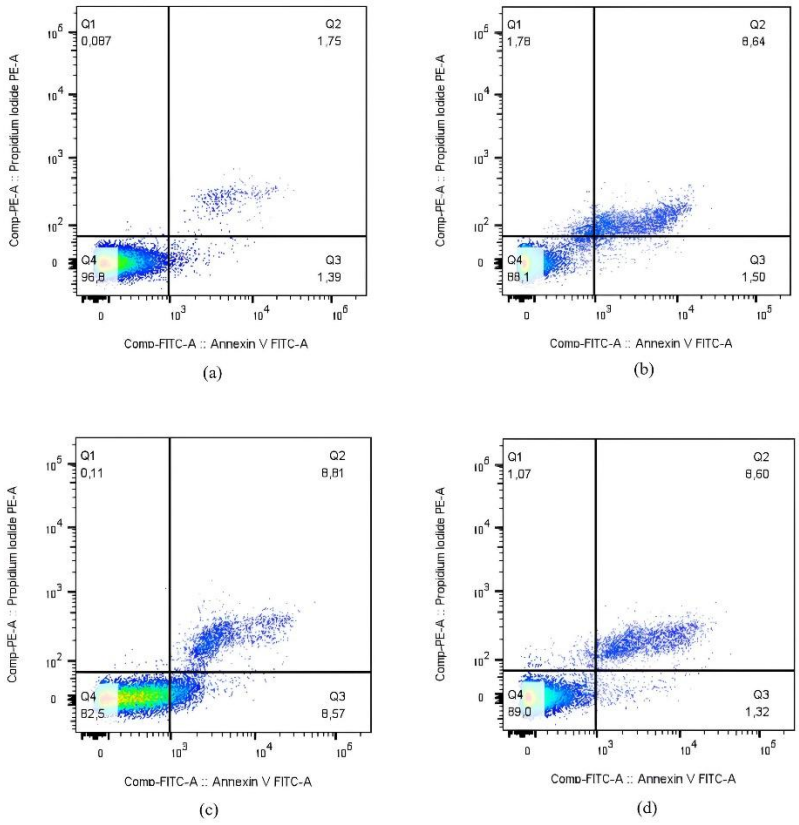

Figure 6 Layout of Q3 fluorescence (Anexin V (+), PI (−)) (a) Control cells, (b) Solvent control, (c) Extract, and (d) Phytosome-Extract against MCF-7 cells.

In MCF-7 cells treated by the extract, the percentage of cells undergoing apoptosis was 6.56%, while those treated by Phytosome extract was 1.23%. It can be seen that treatment of extract had increased the number of cells undergoing apoptosis compared to cell control and solvent control. Administration of Phytosome extract did not significantly increase compared to control, both cell control and solvent control. The treatment groups experiencing late apoptosis were the solvent control group (7.73%) and Phytosome (8.12%). There was no necrosis mechanism in each test group. The number of living cells in each group was relatively the same between 95 - 87%.

According to research [62], MCF-7 cells induced by staurosporine, there was an increase in annexin-V binding in MCF-7 cells after 4 h of treatment and increased with increasing time. Compared to HEK 293-A cells which showed results after 14 h. The low number of living cells can be caused by too long exposure. Where MCF-7 cells can be detected at 4 h, so that with too long exposure the number of dead cells will increase. The extract showed an apoptosis mechanism with the annexin V method while phytosome experiences late

apoptosis. The concept of annexin V labeling involves the staining of phosphatidylserines (PS) located on the outer membrane of cells. Early apoptotic cells have phosphatidylserine on the external surface of the plasma membrane. Phosphatidylserine can be marked by annexin V labeling. Cells in late apoptosis and necrotic cells will have compromised cell membrane integrity, rendering them susceptible to annexin V staining [63].

The scope of this study was limited to in vitro evaluation of the ethanolic extract of *Zanthoxylum acanthopodium* in phytosomal form, including its characterization and cytotoxic effects against MCF-7 and HEK293-A cells. This study did not include in vivo experiments, pharmacokinetic profiling, or the identification of specific active constituents responsible for the cytotoxic effects. Further investigations are needed to validate these findings in animal models and to optimize the formulation for improved selectivity and targeted delivery.

Despite the enhanced physical stability and entrapment efficiency of the phytosomal formulation, no significant increase in cytotoxicity or selectivity was observed compared to the crude extract. This may be due to the neutral charge and non-targeted nature of the phosphatidylcholine-based vesicles, which are internalized by cells through passive endocytosis without discrimination between normal and cancerous cells. Previous studies on phytosomes with modified surface properties or conjugated ligands have shown better pharmacodynamic outcomes. Therefore, further enhancement—such as incorporating cancer-targeting moieties or using standardized phytochemical fractions—is essential to fully realize the therapeutic potential of *Z. acanthopodium* phytosomes

Conclusions

The ethanolic extract of *Zanthoxylum acanthopodium* demonstrated cytotoxic activity against MCF-7 breast cancer cells, and its formulation into a phytosomal system improved physical stability and entrapment efficiency. However, the phytosome did not significantly enhance cytotoxic potency or selectivity compared to the crude extract. The low selectivity index indicates non-specific toxicity, likely due to the absence of targeted delivery mechanisms and the complexity of the crude extract. Future studies should focus on isolating specific active compounds, incorporating targeting ligands (e.g., folate, peptides), or applying PEGylation to improve circulation time and tumor specificity. In vivo evaluation and pharmacokinetic profiling are also warranted to validate the formulation's clinical potential.

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