Anticancer Activity from Stem Bark of *Nauclea subdita* Nanoliposomes Extract on MCF-7 Breast Cancer Cells: *In vitro* and *In silico* Study

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Abstract

Breast cancer caused many deaths in Indonesia in 2022, and although chemotherapy is commonly used for treatment, it often leads to serious side effects. Natural alternatives such as Bangkal (*Nauclea subdita*), which contains alkaloids with potential anticancer properties, may offer milder side effects. Studies have shown that Bangkal (*Nauclea subdita*) stem bark extract can inhibit the growth of cancer cells like LNCaP and PC-3. However, natural compounds usually face challenges such as low bioavailability and poor solubility, which can limit their effectiveness. This study investigates alkaloids in Bangkal (*Nauclea subdita*) stem bark as anti-breast cancer agents targeting MCF-7 cells and develops them into nanoliposome form to improve their effectiveness. A total of 960 grams of Bangkal (*Nauclea subdita*) stem bark powder was macerated with n-hexane, followed by dichloromethane extraction after being treated with ammonia solution. The extracts were analyzed using LC-MS. Nanoliposomes were prepared via thin-layer hydration and characterized using PSA. *In vitro* tests included MTT and co-combination assays, while molecular docking was used for *in silico* analysis. Results indicate that the alkaloids atropine, funtumine, vasicinol, oxymatrine, and evodiamine effectively inhibit Bcl-2 protein expression more than HER2. The optimal nanoliposome formula (F3) had a particle size of 148 nm, PDI of 0.673, and zeta potential of -41.3. The IC_{50} value of the crude extract (361.5 µg/mL), initially categorized as weak, improved to a moderate level (188.18 µg/mL) after formulation into a nanoliposome preparation. These findings indicate that Bangkal (*Nauclea subdita*) stem bark can be developed into a nanoliposome formulation and shows potential as an anticancer agent based on both *in vitro* and *in silico* studies.

Keywords: Nauclea subdita, *alkaloids, nanoliposome, breast cancer, MCF-7.*

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INTRODUCTION

Conventional cancer treatment strategies generally involve surgery, chemotherapy, radiation therapy, hormone therapy, or a combination of these modalities (Deswita & Ningseh, 2023). Although chemotherapy remains a cornerstone of oncological care, its clinical efficacy is frequently constrained by an inability to achieve durable responses in metastatic solid tumors, which often manifest as partial and highly heterogeneous outcomes. Severe systemic toxicities and the emergence of multifaceted resistance mechanisms further compromise its therapeutic utility. These include intrinsic or acquired genetic mutations, the upregulation of drug efflux pumps that diminish intracellular drug concentrations, and enhanced DNA repair pathways that mitigate chemotherapy-induced damage. Furthermore, as many cytotoxic agents preferentially target rapidly proliferating cells, quiescent cancer stem cells, characterized by slow division rates and robust repair systems, frequently evade eradication. This therapeutic challenge is compounded by stochastic variability in protein expression and the potential for reversible senescence, both of which facilitate treatment evasion and subsequent disease relapse (Tilsed, *et al*., 2022).

The use of herbal medicine offers a promising alternative for cancer therapy, with several advantages, including accessibility, natural origin, lower toxicity, reduced side effects, and cost-effectiveness (Regassa, *et al*., 2022). One potential natural source is the Bangkal tree (*Nauclea subdita* (Korth.) Steud.). According to Liew, *et al*. (2014), five alkaloid compounds were isolated from the stem bark of Bangkal (*Nauclea subdita*), a plant found in Malaysia. *In vitro* identification revealed five alkaloids, four of which had previously been identified, including Angustoline, Angustidine, Angustine, Nauclefine, and Subditine. These compounds were tested *in vitro* on LNCaP and PC-3 prostate cancer cell lines. Using the MTT assay with paclitaxel as a comparative chemotherapeutic agent, Subditine exhibited the highest inhibitory activity among the five isolated alkaloids, with IC_{50} values of 4.04 µg/mL against LNCaP cells and 4.6 µg/mL against PC-3 cells. Notably, Subditine demonstrated higher selectivity toward LNCaP and PC-3 cells than toward normal RWPE-1 cells, with its IC_{50} increasing. In contrast, paclitaxel showed lower selectivity, with IC_{50} values of 1.08 µg/mL, 1.13 µg/mL, and 1.34 µg/mL for LNCaP, PC3, and RWPE-1, respectively. Furthermore, Western blot analysis indicated that Subditine promotes apoptosis by downregulating the expression of Bcl-2 and Bcl-xl (Liew, *et al*., 2014).

Another finding by Liew, *et al*. (2014) was Angustoline, another compound found in Bangkal (*Nauclea subdita*) stem bark, which was independently tested as a pure compound by Li, *et al*. (2020). The results showed that Angustoline effectively inhibits the growth of esophageal cancer cells. It demonstrates the ability to upregulate Liver Kinase B1 (LKB1) and AMP-Activated Protein Kinase (AMPK), where LKB1 mediates AMPK activation to suppress cancer cell proliferation. Moreover, Angustoline inhibits ELAV-like family member 1 (ELAVL1) and Lipoma-Associated Protein 2 (LPACT2), two proteins that otherwise promote cancer cell survival and evade apoptosis.

Despite their potential, administering alkaloids as anticancer agents often faces challenges, such as poor specificity for cancer cells. To resolve this, nanocarrier based strategies are viable, particularly through the formulation of extract loaded nanoliposomes. Lipid based nanocarriers are selected for their ability to solubilize, encapsulate, and deliver alkaloids in a programmed manner, thereby enhancing aqueous solubility, bioavailability, and anticancer efficacy (Loh, *et al*., 2021).

Therefore, this research aims to analyze the alkaloid compounds from Bangkal stem bark (*Nauclea subdita* (Korth.) Steud.) as anticancer agents against MCF-7 breast cancer cells using *in*

vitro and *in silico* approaches, with a nanoliposome formulation. This study is expected to serve as a reference for developing effective and safe alternative treatments for breast cancer using herbal resources.

MATERIAL AND METHODS

Determination

Plant determination tests were conducted at the Faculty of Biology, Ahmad Dahlan University, Yogyakarta. These test results indicated that the sample was indeed a bangkal tree plant (*Nauclea subdita* (Korth.) Steud.).

Material

The stem bark of *Nauclea subdita* was collected from Tabalong, South Kalimantan. The specimen was identified at the Faculty of Biology, Ahmad Dahlan University, Yogyakarta, with the number 060/Lab.Bio/B/II/2024. The collected material was dried in an oven at 50°C until fully desiccated. Subsequently, the dried bark was pulverized using a blender to obtain a fine powder.

Extraction

The extraction procedure was adapted from the method described by Liew, *et al*. (2014), with specific adjustments to the *Nauclea subdita* powder samples used in this study, while maintaining the original powder-to-solvent ratio of 1:10:10 (*Nauclea subdita* powder:n-hexane:dichloromethane). In this study, 690 g of *Nauclea subdita* stem bark powder was macerated with 6.9 L of n-hexane for 3 days at room temperature. Following this, the extract was filtered, and the remaining residue was air-dried at room temperature. The dried residue was then basified by moistening it with ammonia for two hours to facilitate alkaloid liberation. Subsequently, a secondary extraction was performed using 6.9 liters of dichloromethane (CH2Cl2), conducted twice over a period of 3 days.

Compound Content Test LC-MS Method

LC-MS was used to analyze alkaloid metabolites in *Nauclea subdita* stem bark extract. Liquid chromatography was performed on a Thermo Scientific™ Accucore™ Phenyl-Hexyl column with a gradient mobile phase: 0.1% formic acid in water with 5 mM ammonium acetate (A) and 0.1% formic acid in MeCN (B). The flow rate was 0.2 mL/min, the column temperature was 40°C, and the injection volume was 3 µL. MS/MS was conducted in positive-ion electrospray mode, optimizing capillary voltage (3.0 kV), source temperature (150°C), desolvation temperature (400°C), cone gas (100 L/h), and desolvation gas (300 L/h) (Hajrulai, *et al*., 2022). The LC-MS results are a chromatogram for each analyte and standard used. In qualitative data, an analyte is described in a chromatogram based on a retention time specific to the channel, with the m/z ratio determined (Harmita, *et al*., 2019).

Synthesis of Bangkal (*Nauclea subdita*) Stem Bark Extract Nanoliposome

Formulations were prepared using the thin-layer hydration technique, utilizing the materials and quantities specified in the Table. 1.

Organoleptic Evaluation and pH measurement

The evaluation test was conducted as a visual organoleptic test and a dosage form test. Furthermore, the pH test was performed using a pH meter (Listiyana, *et al.*, 2020).

Nanoparticle Characterization Test with Particle Size Analyzer (PSA) Instrument

A particle size analyzer (PSA) was used in this study to determine the particle size and particle size distribution of nanoliposomes derived from Bangkal (*Nauclea subdita*) bark extract. PSA measurements were performed using a sample that had been homogenized using a sonicator operating in PBS. Determination of particle size, polydispersity

Table 1. Formulation of bangkal (*Nauclea subdita*) nanoliposome extract.

Material	Composition			Function
	F1	F2	F3	
Bangkal (*Nauclea subdita*) stem bark extract	350 mg	350 mg	350 mg	Active compound
Soy lecithin	800 mg	675 mg	300 mg	Bilayer forming
Tween 20	1.600 mg	1.350 mg 6	00 mg	Stabilizer
Cloroform	300 ml	250 ml	150 ml	Soy lecithin solvent
PBS pH 5.7	Ad 100 ml	Ad 100 ml	Ad 100 ml	Solvent

index (PDI), and zeta potential using the PSA instrument involves adding 3 mL of nanoliposomes to a cuvette, then inserting the cuvette into the PSA instrument holder (Munawiroh, *et al*., 2018).

Cytotoxic Test with MTT Assay

The sterilization of experimental instruments was conducted by washing with 1% Teepol, immersion in disinfectants (alcohol, Preptol, and HiBiCEPT) for 15 minutes, followed by steam sterilization via autoclaving at 121°C for 15 minutes, and subsequent hot-air oven drying. Complete culture media was prepared by supplementing Rosewell Park Memorial Institute (RPMI) 1640 medium with 10% Fetal Bovine Serum (FBS), 0.5% Fungizone, and 1% penicillin-streptomycin, noting that the use of phenol-red-free media during MTT incubation can minimize potential optical interference. For sample preparation, 5 mg of Bangkal (*Nauclea subdita*) stem bark extract was dissolved in 50 µL of DMSO to achieve a 100,000 ppm primary concentration, which was then diluted to a 10,000 ppm stock solution and further processed into a concentration series of 500, 250, 125, 62.5, and 31.25 µg/mL for both the crude extract and nanoliposome formulations. MK cells were seeded into 96-well plates at a density of 5×10^3 cells per 100 µL, with three wells designated as media controls. Maintaining a precise seeding density is critical, as excessive confluence can lead to metabolic shifts or nutrient depletion that may confound the resulting optical density (OD) values. After a 24 h incubation period, the media was aspirated, the cells were washed with Phosphate-Buffered Saline (PBS), and 50 µL of culture media containing either 0.2% DMSO (vehicle control) or the nanoliposomized extract was added in triplicate. Following an additional 24 h incubation, the MTT assay was performed by adding 10 µL of a 0.5 mg/mL MTT reagent to each well.

While standard protocols vary, an incubation period of 2 to 4 h is generally optimal to prevent reagent-induced cytotoxicity while allowing for the formation of purple formazan crystals, which serve as indicators of mitochondrial metabolic activity in viable cells. These crystals were subsequently observed under an inverted microscope and solubilized using a suitable solvent, after which the absorbance was measured at 595 nm using an ELISA plate reader. It is important to recognize that the measured OD reflects a composite of cell number, metabolic rate, and the efficiency of MTT cellular uptake, requiring the results to be interpreted as relative metabolic activity relative to the control group to determine the cytotoxic potential of the formulations accurately (Ghasemi, *et al*., 2021).

Combination Test of Bangkal (*Nauclea subdita*) Stem Bark Extract Nanoliposome with Doxorubicin

Cells ($5x10^3$ cells/100 µL MK) were transferred into 96-well plates, with control media in three wells. After a 2 h recovery, a series of nanoliposomal bark extract and doxorubicin concentrations (½, ¼, and 1/8 IC_{50}) were prepared. Absorbance at 595 nm was measured with an ELISA reader, and live cell percentage and Combination

Index (CI) were calculated (CCRC, 2010). The results indicated either antagonistic or synergistic interactions. The combination index score, generated by averaging across concentrations, helps train models by addressing multiple local minima and maxima in the dose-response surface (Preuer, *et al*., 2017).

In Silico Analysis

Ligand preparation

Ligands consisting of alkaloid compounds identified from *Nauclea subdita* stem bark through LC-MS screening were obtained and prepared before docking analysis. The ligand structures were initially retrieved or generated in .sdf format and subsequently converted into .pdb format using Open Babel. Geometry optimization was performed when necessary to obtain stable conformations. Furthermore, ligand files were converted to.pdbqt format using MGLTools to meet the docking process requirements. Final ligand preparation, including the assignment of charges and rotatable bonds, was conducted using AutoDockTools.

Protein Preparation

The target proteins used in this study were Bcl-2 (PDB ID: 6O0K) and HER2 (PDB ID: 3PP0), obtained from the Protein Data Bank. The Bcl-2 structure has a resolution of 1.62 Å and contains venetoclax (LBM) as its co-crystallized native ligand. In comparison, the HER2 structure has a resolution of 2.25 Å with 03Q as its co-crystallized ligand. These co-crystallized ligands were used to define the active binding sites and support the validation of the docking protocol. In addition, doxorubicin was used as a reference in this research due to its established clinical use in cancer therapy. Although it does not specifically target Bcl-2 or HER2, it serves as a benchmark for comparing the binding affinity of the tested compounds with that of a standard chemotherapeutic agent. Prior to docking, the protein structures were prepared by removing water molecules and other non-essential heteroatoms using BIOVIA Discovery Studio. Native ligands and other irrelevant molecules were separated from the receptor to ensure accurate docking simulation. Subsequently, hydrogen atoms were added, and Gasteiger charges were assigned using AutoDockTools. Finally, the prepared protein structures were converted into.pdbqt format using MGLTools for further docking analysis.

Analysis of Cytotoxic Test MTT Assay

The data is obtained as an absorption from the ELISA reader and will be converted into a live percentage with the help of a formula:

$$\frac{(\text{Treatment Absorbance} - \text{Control Media Absorbance})}{(\text{Cell Control Absorbance} - \text{Control Media Absorbance})} \times 100\%$$

Analysis of Combination Test Bangkal (*Nauclea subdita*) Stem Bark Extract Nanoliposome and Doxorubicin

The Combination Index (CI) analysis produces a quantitative parameter score that describes the effectiveness of the combination using the equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

Dx : Concentration of a single compound required to produce an effect equivalent to that of the combination.
$(D)_1$, $(D)_2$: Concentrations of the two compounds required to produce the same effect.

The CI values calculated using the formula were interpreted according to the criteria presented in Table 2.

Table 2. Interpretation of the Combination Index.

CI Value	Interpretation
< 0.1	Very strong synergistic effect
0.1-0.3	Strong synergistic effect
0.3-0.7	Synergistic effect
0.7-0.9	Mild to moderate synergistic effect
0.9-1.1	Nearly additive effect
1.1-1.45	Mild to moderate antagonistic effect
1.45-3.3	Antagonistic effect
>3.3	Strong to very strong antagonistic effect

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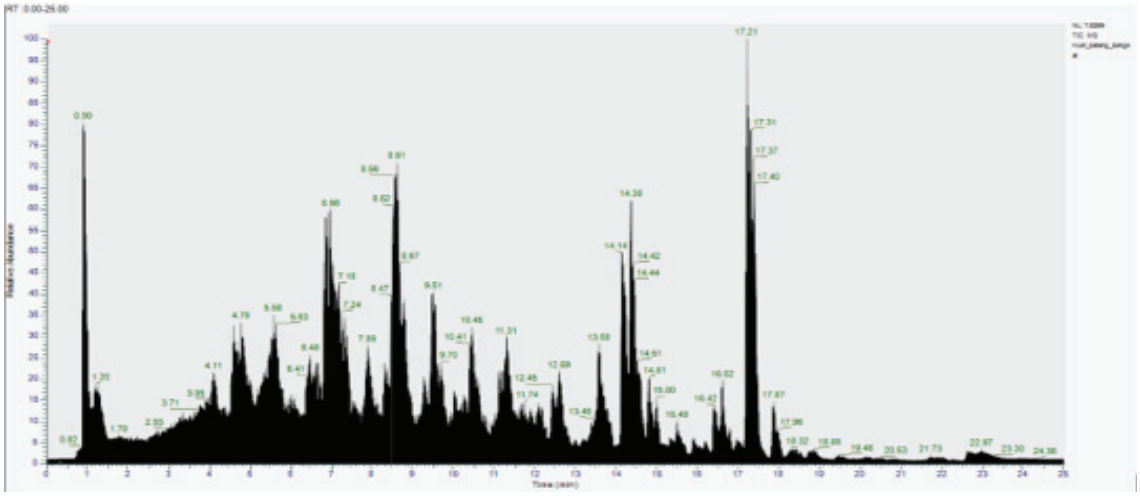


Figure 1. Peak Chromatogram of bark extract by LC-MS.

Molecular Docking Analysis

A molecule is said to have a good stability affinity if the RMSD is <2 Å. The more negative the free binding energy, the higher the ligand affinities (Susilo, *et al*., 2023). RMSD used to validate a docking method using AutoDock software. This validation is performed on valid ligands and docking results. The Root Mean Square Deviation (RMSD)value is a parameter used in this validation.

Table 3. Analysis results of alkaloid compounds using LC-MS.

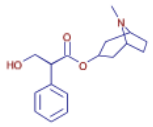
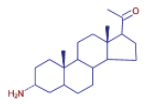
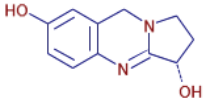
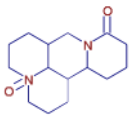
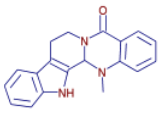
Name of compound	Chemical formula	Retention time (Rt)	Composition (%)	Structure
Atropine	$C_{17}H_{23}NO_3$	6.651	0.26	
Funtumine	$C_{21}H_{35}NO$	11.026	0.085	
Vasicinol	$C_{11}H_{12}N_2O_2$	3.092	0.082	
Oxymatrine	$C_{15}H_{24}N_2O_2$	7.573	0.043	
Evodiamine	$C_{19}H_{17}N_3O$	6.970	0.032	

Table 4. Results of nanoliposome physicochemical evaluation and PSA test results.

	Color	Smell	Form	pH	Particle size	Polydispersity index	Zeta Potential
F1	Light orange	Pungent	Light viscosity	6.76	297.3 nm	197.8	26 mv, negative polarity
F2	Light orange	Pungent	Light viscosity	6.51	197.8 nm	0.854	1.0 mv, negative polarity
F3	Light orange	Pungent	Light viscosity	6.97	148 nm	0.673	41.3 mv, negative polarity

RESULTS

Extraction

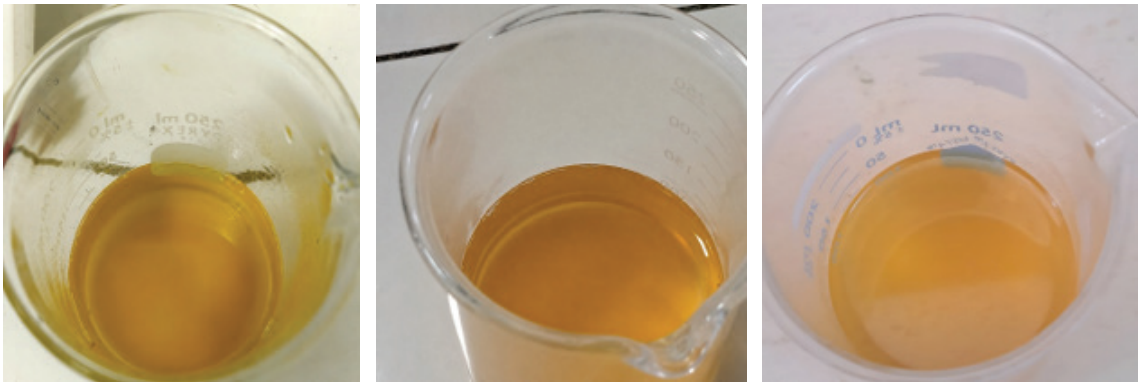
From the extraction process, 9.665 liters of liquid extract were obtained. The liquid extract was concentrated to a thick extract, yielding 8.8613 grams (1.284%).

LC-MS Analysis of Bangkal *(Nauclea subdita)* Stem Bark Extract

Figure 1 shows the peak chromatogram results of the LC-MS test that has been carried out on the identification of bark extract. In the Bangkal (*Nauclea subdita*) stem bark extract, 340 compounds have been identified. Although LC-MS analysis revealed a complex chemical profile comprising 340 compounds, they were highly diverse in terms of chemical classes and biological relevance. Among them, alkaloids were specifically prioritized due to their well-documented pharmacological activities, particularly their anticancer potential. As demonstrated in this study, after interpretation, there are five alkaloid compounds which, in previous studies, have been shown to have anticancer mechanisms according to (Table 1).

Integrated Synthesis and Physicochemical Characterization of Bangkal (*Nauclea subdita*) Extract Nanoliposomest

Three nanoliposome formulations containing 0.35% extract were successfully prepared using different ratios of soy lecithin and Tween 20, namely F1 (800 mg:1600 mg), F2 (675 mg:1350 mg), and F3 (300 mg:600 mg). Following the synthesis, physicochemical characterization was performed to evaluate the formulation's properties.



A B C

Figure 2. (A) Formula 1 of *Nauclea subdita* stem bark nanoliposome extract. (B) Formula 2 of *Nauclea subdita* stem bark nanoliposome extract (C) Formula 3 of *Nauclea subdita* stem bark nanoliposome extract.

This test included organoleptic assessment focusing on color, odor, and physical form, as well as pH measurement. The results are shown in Table 4.

Based on the physicochemical evaluation of Bangkal (*Nauclea subdita*) bark extract in the form of nanoliposomes, all formulations were found to have the same color, odor, and consistency. In addition to these parameters, solubility was qualitatively evaluated by visual observation as part of the physicochemical characterization. This evaluation included a thorough examination of the clarity and homogeneity of each formulation. As shown in Figure 2 the results appear clear and homogeneous.

Furthermore, the resulting pH values for each formulation fell within the extracellular pH range characteristic of cancer cells, which typically spans from 6.0 to 7.2. This range is notably more acidic than the physiological pH of normal cells, which is approximately 7.4. By maintaining a pH level compatible with the tumor microenvironment yet remaining relatively close to physiological conditions, these nanoliposome formulations are hypothesized to provide optimal therapeutic efficacy against cancer cells while minimizing adverse effects on healthy tissues (Li, *et al*., 2020).

Particle Size Analyzer (PSA) Test

Nanoliposomes are considered to have a good particle size when they are in the 100-150 nm range. PDI value for standard nanoliposome <0.7, and zeta potential value for the standard <-30 mv or >+30 mv. The PSA test result of the Bangkal (*Nauclea subdita*) nanoliposome extract can be seen in (Table 4).

Bangkal (*Nauclea subdita*) stem bark extract containing alkaloids was formulated into nanoliposomes. Based on the PSA results, only the third formulation met the requisite criteria for nanoliposome standards. All formulas used the same method, differing only in the addition of soy lecithin and tween 20 (Table 1). Table 4 shows that these additives influence particle size, polydispersity index (PDI), and zeta potential, with higher amounts increasing particle size and PI and reducing repulsive forces between particles in the PSA analysis.

Molecular docking

Molecular docking was performed using AutoDock Vina, and the resulting structure was visualized in 2D with DS Visualizer. The molecular docking data are presented in Table 5, Figure 3 and Figure 4.

Table 5. Molecular docking results between ligand and protein (Bcl-2 and HER2).

Name of compound	BCl-2		HER2	
	Docking Score	RMSD	*Docking Score*	RMSD
Doxorubicin	-7.0	1.911	-6.5	1.771
Atropine	-8.8	0.920	-5.0	1.592
Oxymatrine	-8.2	1.077	-4.9	1.943
Normorphine	-8.1	1.418	-5.5	1.808
Evodiamine	-7.5	1.975	-6.1	1.872
Alchornine	-7.0	1.880	-4.3	1.365
Narcissidine	-7.7	1.462	-4.9	1.996
Funtumine	-7.6	1.207	-4.4	1.862
Vasicinol	-7.7	1.687	-4.5	1.625

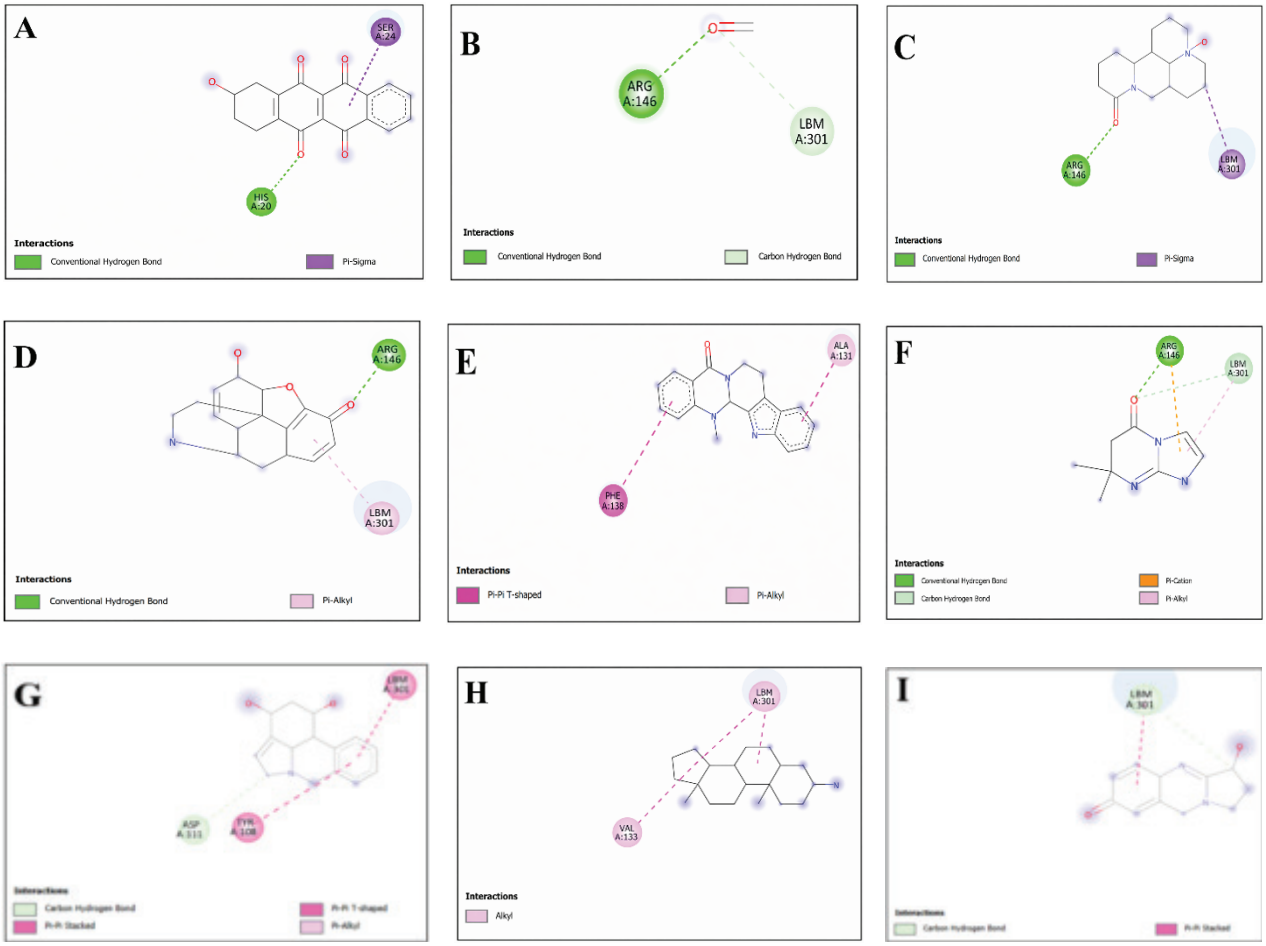


Figure 3. 2D visualization of the interaction between test compounds and the Bcl-2 protein: A. Doxorubicin; B. Atropine; C. Oxymatrine; D. Evodiamine; E. Nirmorphine; F. Alchornine; G. Narcissidine; H. Funtumine; I. Vasicinol.

In the Bcl-2 docking assay, all compounds exhibited negative docking scores, with Atropine demonstrating the highest binding affinity (-8.8 kcal/mol) and the lowest RMSD (0.920 Å), surpassing the control drug Doxorubicin (-7.0 kcal/mol). For the HER2 protein, Doxorubicin showed the strongest affinity (-6.5 kcal/mol), followed closely by Evodiamine (-6.1 kcal/mol; RMSD 1.872 Å). All selected conformations yielded RMSD values below 2 Å, confirming the validity of the docking poses.

The 2D molecular interaction analysis revealed that all tested compounds established diverse non-covalent interactions within the binding pockets of Bcl-2 and HER2, including hydrogen bonds, carbon-hydrogen bonds, and hydrophobic interactions such as π–π stacked and π–alkyl interactions. In the Bcl-2 protein, the reference ligand doxorubicin interacted with His20 and Ser24 through hydrogen bonds, while several test compounds, including Atropine, oxymatrine, evodiamine, and alchorinine, exhibited interactions with the key residue Arg146. Additionally, aromatic residues such as Phe138 and Tyr108 contributed to hydrophobic interactions, which are known to enhance ligand-receptor stability. These findings

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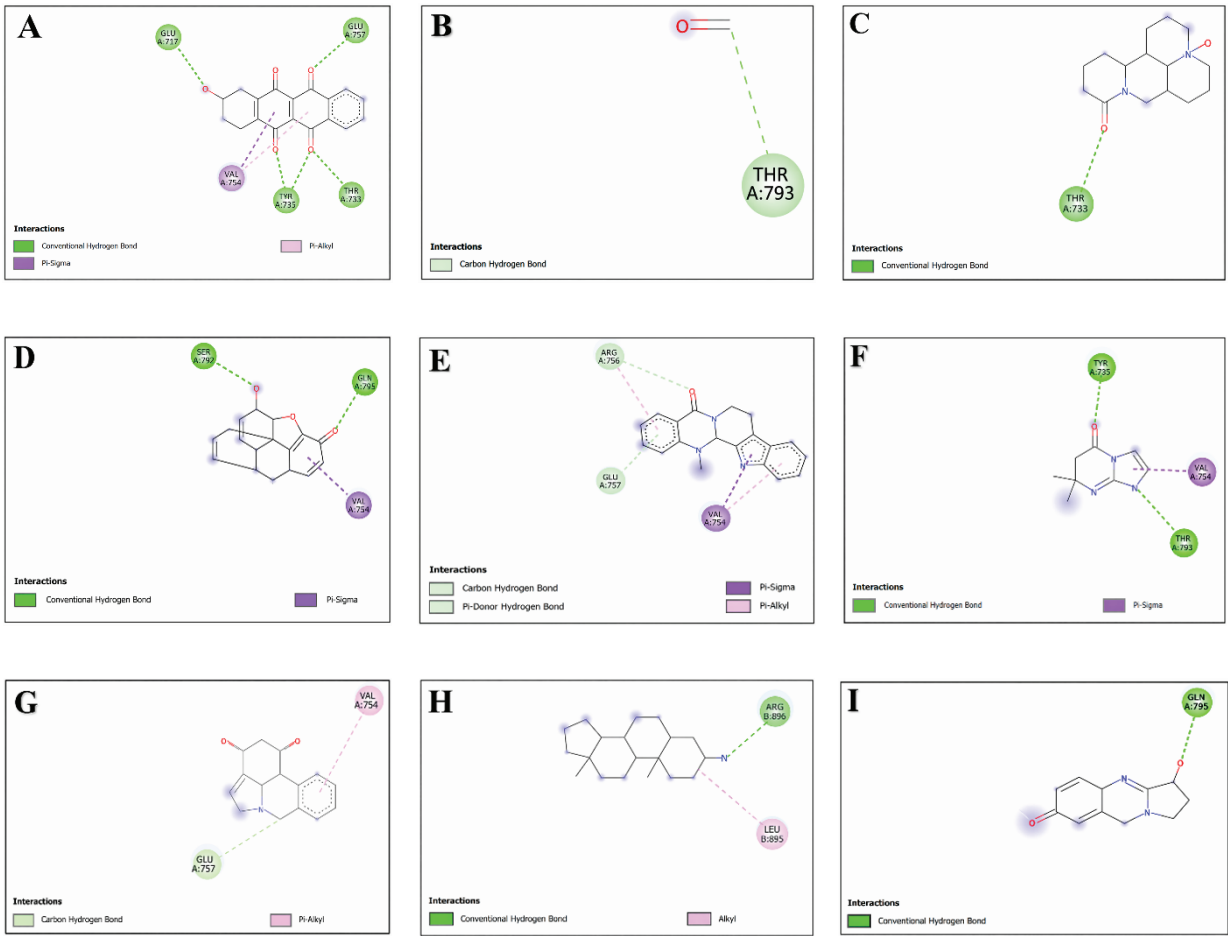


Figure 4. 2D visualization of the interaction between test compounds and the HER2 protein: A. Doxorubicin, B. Atropine, C. Oxymatrine, D. Evodiamine, E. Nirmorphine, F. Alchornine, G. Narcissidine, H. Funtumine, I. Vasicinol.

are consistent with previous studies indicating that both hydrophobic and charged amino acid residues play critical roles in stabilizing ligand binding and influencing binding affinity (Dela Cruz, *et al*., 2022).

The 2D molecular interaction analysis of HER2 protein revealed that doxorubicin formed a network of multiple hydrogen bonds with key residues, including Glu717, Glu757, Thr733, and Tyr735, as well as hydrophobic interactions with Val754. Test compounds such as atropine and oxymatrine exhibited similar binding patterns, particularly involving residues Thr733 and Thr793 situated within the active kinase domain. Meanwhile, nirmorphine, funtumine, and alchorinine utilized a combination of polar and hydrophobic interactions with residues Arg756, Glu757, Tyr735, and Val754. The consistent involvement of these residues underscores their role as crucial binding hotspots, where specific interactions at these active-site residues serve as primary determinants of ligand conformational stability and biological inhibitory efficacy against the HER2 target (Millan-Casarrubias, *et al*., 2023).

Table 6. Cytotoxic results with MTT Assay.

Sample	Equation	IC_{50} (µg/ml)
Bangkal (*Nauclea subdita*) stem bark extract alone	y = -0.1683x + 110.85 R^2 = 0.9898	361.5 (Weak)
Bangkal (*Nauclea subdita*) stem bark extract nanoliposome	y = -0.1837x + 84.569 R^2 = 0.9191	188.18 (Moderate)
Doxorubicin	y = -20.011x + 110.89 R^2 = 0.9593	3.04 (Strong)

Cytotoxic test with MTT assay

Table 6 shows the results of the cytotoxicity test using the MTT assay method performed on MCF-7 breast cancer cells. The IC_{50} value of the *Nauclea subdita* stem bark extract alone increased from weak (361.5 µg/ml) to moderate (188.18 µg/ml) after the bark extract was processed into a nanoliposome preparation.

Combination Test of Bangkal (*Nauclea subdita*) Stem Bark Extract Nanoliposome with Doxorubicin.

The results of the combination test included two endpoints: MCF-7 cell viability and the combination index (CI). Based on the MCF-7 cell viability calculation, this combination test showed good results, with cell viability <50% (Table 7).

Table 7. Viability of MCF-7 cells in combination treatment of nanoliposome extract and doxorubicin.

Concentration of Bangkal (*Nauclea subdita*) nanoliposome extract (µg/ml)		**0**	**94.09**	**47.04**	**23.52**
Concentration of *Doxorubicin* (µg/ml)	**0**	100.0	-0.25	-0.28	43.95
	1.52	26.08	2.13	-0.30	-0.32
	0.76	40.98	0.46	0.31	1.19
	0.38	42.89	0.40	30.90	33.48

Table 8. Combination index of MCF-7 cells on combination treatment of nanoliposome extract and doxorubicin.

Doses combination		**CI**	**Therapy effects**
Bangkal (*Nauclea subdita*) stem bark extract nanoliposome (µg/ml)	**Doxorubicin (µg/ml)**		
94.09	1.52	1.12	Light-medium antagonist
94.09	0.76	0.55	Synergist
94.09	0.38	0.27	Powerful synergist
47.04	1.52	1.94	Antagonist
47.04	0.76	2.00	Antagonist
47.04	0.38	3.37	Powerful antagonist
23.52	1.52	2.00	Antagonist
23.52	0.76	2.02	Antagonist
23.52	0.38	2.12	Antagonist

The value of the combination index analysis of nanoliposomes of Bangkal (*Nauclea subdita*) stem bark extract with doxorubicin shows that, out of 9 combination doses, one combination dose shows a strong synergistic effect (one synergist), one mild-moderate antagonist, five antagonists, and one strong antagonist. Table 8 shows the results of the combination index analysis of bark extract nanoliposomes with doxorubicin.

DISCUSSION

The extraction process in this study was designed to maximize the isolation of alkaloid fractions by converting polar alkaloid salts into their non-polar free base forms using ammonia, facilitating their dissolution in CH2Cl2. Subsequent phytochemical screening via LC-MS identified five distinct alkaloids with known anticancer properties: atropine, funtumine, vasicinol, oxymatrine, and evodiamine. The presence of these compounds likely contributes to the extract's cytotoxic activity. For instance, atropine is known to inhibit muscarinic acetylcholine receptors (mAChRs) expressed in various cancer cells, including breast cancer models (Ahmad, *et al*., 2022). Funtumine, a steroidal alkaloid, exerts antiproliferative effects by modulating mitochondrial pathways and stimulating caspase-3/7 activation (Badmus, *et al*., 2020). Vasicinol shows potential as an NF-κB inhibitor, inducing apoptosis by activating Bax and cleaved PARP (Kumar, *et al*., 2022). Furthermore, oxymatrine inhibits aerobic glycolysis via Pyruvate Kinase M2 (PKM2), thereby hindering cancer cell migration (Li, *et al*., 2022), while evodiamine suppresses cell proliferation and tumor markers such as VEGF by inhibiting key pathways, including Wnt/β-catenin and PI3K/AKT (Li, *et al*., 2022).

In formulations containing nanoparticles, it is essential to perform PSA testing to determine the particle size and size distribution characteristics of the formulation. The characteristics measured in this formulation testing include particle size, polydispersity index, and the zeta potential of the nanoliposomes. These parameters are critical for evaluating liposome quality, as a well-formulated liposomal system should exhibit physicochemical properties that ensure stability, uniformity, and effective biological interactions. Ideally, vesicle size should remain below 150 nm to facilitate efficient cellular uptake, while a low polydispersity index (≤0.30) indicates a monodisperse and uniform particle distribution. Additionally, surface charge plays a crucial role in maintaining colloidal stability; liposomes with an absolute zeta potential greater than ±30 mV are generally considered stable due to the strong electrostatic repulsion that prevents aggregation and enhances redispersibility (Németh, *et al*., 2022). Based on the results of this study, only Formula 3 fulfilled all the nanoliposome characterization standards, including particle size, polydispersity index (PDI), and zeta potential. Interestingly, although Formula 1 exhibited the lowest PDI value, indicating a more monodisperse particle size distribution, it did not meet the required criteria for particle size and zeta potential. This finding suggests that a low PDI alone does not necessarily reflect overall system stability or suitability.

The relatively low PDI observed in Formula 1 can be attributed to the higher Tween 20 concentration, which acts as a surfactant, effectively reducing interfacial tension and promoting uniform particle formation. The increased amount of Tween 20 also enhances surface coverage, thereby minimizing irregular coalescence during sonication. However, excessive surfactant concentration may negatively affect other parameters, such as particle size enlargement and reduced zeta potential stability. In contrast, Formula 3, with the lowest ratio of soy lecithin and Tween 20, demonstrated a more balanced physicochemical profile and successfully met all PSA criteria. It indicates that an optimal lipid to surfactant ratio is crucial for producing stable nanoliposomes. Overall, the differences among the three formulations are

primarily influenced by variations in the soy lecithin to Tween 20 ratio, highlighting the importance of excipient composition in determining nanoliposome characteristics.

In formula 3, a particle size of 148 nm (100–150 nm) was obtained. The Polydispersity Index (PI) value was 0.673 (<0.7). The PI value is required for nanoliposome preparations to assess homogeneity; the lower the PI value, the better the homogeneity. The zeta potential was -41.3 mV. A good zeta potential is less than -30 mV or greater than +30 mV. The zeta potential value describes the repulsive force between particles. The better value of zeta potential, the more stable the dispersion system.

The cytotoxic evaluation using the MTT assay revealed distinct differences in efficacy between the formulations. The *Nauclea subdita* stem bark extract alone exhibited weak cytotoxicity with an IC_{50} of 361.5 µg/ml. In contrast, the nanoliposome-encapsulated extract demonstrated moderate cytotoxicity (IC_{50}=188.18 µg/ml), which was significantly lower than that of the free extract. Doxorubicin served as the positive control, showing strong cytotoxicity with an IC_{50} of 3.04 µg/ml (Table 6). These results support the hypothesis that encapsulation enhances the therapeutic efficacy of alkaloids. The liposomal system likely improves the solubility and delivery of the active compounds, consistent with findings by Loh, *et al*. (2021), who reported that nanocarriers can enhance alkaloid cytotoxicity by significantly reducing IC_{50} values. Morphological observations further confirmed this activity, as treated cells displayed characteristic signs of apoptosis, including shrinkage and shape irregularities.

To assess the potential to reduce chemotherapeutic toxicity, this study evaluated the combination of the nanoliposome extract and doxorubicin. Co-chemotherapy aims to achieve synergistic effects that maximize efficacy while minimizing dosage. Isobologram analysis and the Combination Index (CI) revealed a potent synergistic interaction (Table 8). Specifically, a combination of 94.09 µg/ml of nanoliposome extract ½ IC_{50} and 0.38 µg/ml of doxorubicin 1/8 IC_{50} yielded a CI of 0.27. A CI value below 1.0 indicates synergism, suggesting that the nanoliposome extract significantly enhances the sensitivity of MCF-7 cells to doxorubicin. Such combination therapies are clinically significant, as they may improve patient survival by reducing overall drug burden and associated adverse effects (Rønneberg, *et al*., 2023). Additionally, this synergistic approach may reduce the likelihood that tumor cells will develop resistance to standard chemotherapeutic agents (Rudolph, *et al*., 2021).

The *in silico* study used AutoDock Vina to examine interactions between test compounds and proteins 6O0K and 3PP0, with all receptors showing RMSD values below 2 Å, ensuring reliable docking. LC-MS identified alkaloids such as atropine, oxymatrine, normorphine, evodiamine, narcissidine, funtumine, and vasicinol demonstrated stronger binding to the Bcl-2 protein than doxorubicin, with energies as low as -7.0 kcal/mol, indicating easier binding and spontaneous inhibition. These findings are further supported by the 2D interaction analysis, which revealed that several compounds form stable interactions with key residues such as Arg146, as well as hydrophobic contacts with aromatic residues, including Phe138 and Tyr108. The presence of both hydrogen bonding and hydrophobic interactions suggests a synergistic contribution to binding stability, reinforcing the observed high binding affinity.

The selection of Bcl-2 as the primary molecular target in this *in silico* study was substantiated by prior findings from Liew, *et al.* (2014), which established that alkaloids from *Nauclea subdita* exert anticancer activity primarily by inducing apoptosis. Bcl-2 functions as a critical anti-apoptotic protein, its overexpression is frequently observed in breast cancer oncogenesis, allowing tumor cells to evade cell death and maintain uncontrolled proliferation (Qian, *et al.*,

2022). The consistent involvement of Arg146 as a key interaction residue across multiple ligands further highlights its role as a critical anchoring site, suggesting that these compounds may effectively disrupt Bcl-2-mediated survival pathways. In contrast, interactions with HER2 did not show more negative values than doxorubicin (-6.5 kcal/mol), although evodiamine reached -6.1 kcal/mol in one conformation. The 2D interaction profiles indicate that, while the compounds interact with important residues such as Thr733, Thr793, Tyr735, and Val754 within the HER2 active site, these interactions are comparatively weaker. These differences likely stem from the alkaloids' specific mechanisms, which enhance apoptosis by favoring a higher affinity for Bcl-2.

As a crucial next step, it is essential to conduct follow-up experimental validations. Specifically, future research should employ immunocytochemical assays to directly observe and quantify the expression of Bcl-2 and HER2 proteins following treatment. This step is vital to confirm whether the observed cytotoxicity is indeed due to the inhibition or downregulation of these specific targets. Such objective data will bridge the gap between computational predictions and biological reality, providing a clearer pathway for developing *Nauclea subdita* alkaloids into standardized phytopharmaceuticals for breast cancer therapy.

CONCLUSION

LC-MS identified alkaloid compounds in *Nauclea subdita* stem bark extract, and PSA testing determined that Formula 3 was the optimal nanoliposome formulation. These alkaloids exhibited a stronger binding affinity for Bcl-2 over HER2, with *in silico* docking values surpassing those of doxorubicin. MTT assays showed moderate cytotoxicity for the nanoliposome extract. At the same time, combination testing with doxorubicin showed a synergistic effect, most notably, 94.09 µg/ml extract (½ IC_{50}) with 0.38 µg/ml doxorubicin (1/8 IC_{50}) yielded a combination index of 0.27.

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