Co-Chemotherapy Effect of Glycosylated Nanoalbumin Genitri Seed Extract Targeting Induced Apoptotic on Overexpressed HER2+ Breast Cancer

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Abstract

HER2-targeted therapy aims to stop proliferation and induced apoptosis. Genitri seeds show anticancer effects and have potential as co-chemotherapy. To maximize bioactive delivery, a glycosylated-nanoalbumin delivery system is used. This research aims to explore the ability of Glycosylated-Nanoalbumin Genitri Seeds (GN-GSE) to induce apoptosis in MCF-7/HER2 cancer cells. The cytotoxic test using the MTT assay showed GSE selectivity (SI=2.7) against cancer cells (IC_{50} 104 µg/mL) and non-toxicity against normal cells (IC_{50} 284 µg/mL). Induction of apoptosis occurs through inhibition of the CDK1 protein which is predicted by molecular docking. GSE has the potential as a synergistic co-chemotherapy with tamoxifen (CI<1) and has good affinity for the CDK1 inhibitory domain. Elaeocarpenine is known to have good affinity with a greater ΔG value (-11.40 kcal/mol) than the native ligand. The GN-GSE formula meets the criteria for nanoparticles with good stability. This research shows that GSE has anti-cancer activity, making it potential as a therapy for HER2 overexpressed breast cancer as well as in a glycosylated nanoalbumin drug delivery system.

Keywords: *Apoptotic induced, co-chemotherapy, genitri seeds, glycosylated, nanoalbumin.*

INTRODUCTION

In 2020, breast cancer was the most prevalent cancer in Indonesia, affecting 30.8% of cancer patients, with nearly 20% of these cases involving HER2+ overexpression (GCO, 2020; Martinez-Saez and Prat, 2021). HER2+ overexpressed breast cancer is a specific subtype characterized by the overexpression of the human epidermal growth factor receptor-2 (HER2+), which contributes to more rapid growth and metastase compared to other types of breast cancer (Novitasari, *et al*., 2023). Unfortunately, inadequate treatment of this malignancy can lead to increased severity, recurrence, and even mortality. Therefore, targeted therapies focusing on HER2 are

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a valuable approach, as they play an anti-apoptotic role by activating ERK, which regulates apoptosis (Amtiria and Berawi, 2018). Consequently, inducing apoptosis is a crucial goal in the treatment of HER2+ overexpressed breast cancer.

To achieve better clinical outcomes in cancer therapy, combination of herbal treatments become favorable, driven by the side effects and complications of long-term drug therapies like tamoxifen. This approach, known as co-chemotherapy, allows for reduced doses while minimizing side effects (Zulfin, *et al*., 2021). One notable local wisdom from Malanesia is genitri (Elaeocarpus ganitrus), often called the "King of Herbal Medicine" for its analgesic, antibacterial, antifungal, and anticancer properties (Mahajanakatti, *et al*., 2022). The alkaloid compound Elaeocarpenine in genitri seeds contributes to its anticancer effects (Primiani, *et al*., 2022). Furthermore, genitri seed extract exhibits cytotoxic effects on PC-3 cancer cells, which can be enhanced by nanoparticle delivery system (Vinay, *et al*., 2021).

The selection of a cancer drug delivery system considers both aspects of selectivity and bioavailability, particularly for non-polar compounds like Elaeocarpenine (log*P* value 3.1 considered as lipophilic compounds) (Vinay, *et al*., 2021; PubChem, 2025). Therefore, glycosylated drug delivery nanoalbumin formula was chosen because it enhances both properties. Glycosylated drug delivery employs glycosylation as a delivery vehicle to target GLUT receptors on the cell surface. Cancer cells often overexpress glucose receptors to obtain energy for metabolism and growth, allowing selective drugs to target cancer cells more effectively than normal cells (Costa, *et al*., 2020). Nanoalbumin acts as a bioactive carrier to improve cellular entry and bioavailability (Li, *et al*., 2015). This study aims to determine if Glycosylated Nanoalbumin from Genitri Seeds (GNGSE) can enhance cytotoxicity against HER2+ overexpressing breast cancer, supporting its role as an apoptosis-inducing co-chemotherapy agent.

MATERIALS AND METHODS

Location and Time of Research

The research was conducted for 4 months in the Pharmaceutical Laboratory and Pharmaceutical Chemistry Laboratory of the Faculty of Pharmacy, as well as the Parasitology Laboratory of the Faculty of Medicine, Public Health, and Nursing (FKKMK) UGM.

Tools and Materials

The tools used include magnetic stirrer (Scientific), Buchner flask (Iwaki), rotary shaker (Stuart), 1-20 µL micropipette (Gilson, Middleton, WI, USA), 100-1000 µL micropipette (Gilson), 96-well plate (Corning), MOE 2010 software, ultraturrax (IKA T50), Brookfield viscometer (LAMY Reology), pH-meter (Hanna), and Particle Size Analyzer (Malvern). The materials used include genitri seeds (Kebumen, Central Java, Indonesia), BSA (Sigma-Aldrich, St. Louis, Missouri), PierceTM reagent (Thermo-scientific, Waltham, MA, USA), aquadest (Waterone), methanol p.a (Merck, Darmstadt, Germany), silica plate 60 (Sigma-Aldrich), Dragendorff reagent (Merck), anisaldehyde-H_2SO_4 (Merck), citroborate (Merck), ethyl acetate (Merck), hexane (Merck), acetic acid (Merck), dichloromethane (Merck), MTT reagent (Sigma-Aldrich), Tamoxifen (Sigma-Aldrich), MCF7/HER2 cell-lines (ATCC), Vero cell-lines (ATCC), DMEM-high glucose (Gibco), fetal bovine serum (Gibco), Penicillin Streptomycin (Gibco), Trypsin 0.25% (Gibco), sodium dodecyl sulfate (Sigma-Aldrich).

Method of Collecting data

Genitri Extraction and Phytochemical Profiling Identification

The genitri seed extraction method was adapted from Primiani, *et al*. (2022), which employed maceration techniques using methanol

p.a for 24 h (solid: liquid 1:10) to obtain GSE. The solvent evaporated with rotary evaporator. Phytochemical profiling with Thin Layer Chromatography (TLC) used a mobile phase of hexane: ethyl acetate: acetic acid (55%:25%:20%). The major alkaloid compound, elaeocarpenine, found in GSE was detected by the Dragendorff reagent (Gowthama, *et al*., 2020).

Cytotoxicity Test with MTT Assay

Cells were seeded in 96-well plates at a density of $5x10^3$ cells/well. 5 mg of GSE was dissolved in 50 µL of DMSO. Tamoxifen was prepared in three concentration series: 0.05 µM, 0.1 µM, and 0.2 µM. The sample solutions were added to the wells and incubated for 24 h, after which MTT reagent was treated. After formazan was formed, a 10% SDS solution was added to stop the reaction, and the absorbance was measured using an ELISA reader at a wavelength of 595 nm. The IC_{50} value was calculated based on linear regression of the percentage of cell viability against the GSE concentrations (Ikawati, *et al*., 2020).

Molecular Docking with MOE

Molecular docking was performed to explore the apoptosis mechanism and signaling proteins involved. The target protein (CDK1) file complexed with native ligand was downloaded from PDB (https://www.rcsb.org/). The docking process was performed with the default MOE 2010. Compounds were subjected to structural visualization with output in the form of ΔG (docking score) indicating the strength of the ligand-receptor interaction (Lestari and Utomo, 2022).

Glycosylated-Nanoalbumin Genitri Seed Extract (GN-GSE) Formulation

Albumin (BSA) was conjugated with fructose in a 1X PBS buffer (5 mL) at a ratio of 2:3 and stirred at 200 rpm. The mixture was then freeze-dried, following the method described by Asyhari, *et al*. (2018). To prepare GN-GSE, the freeze-dried samples were oven-dried at 50°C for durations of 60, 90, and 120 minutes. The resulting samples were dissolved in NaCl at pH 7.4.

Table 1. Glycosylated albumin and GN-GSE preparation formulation.

Material	Weight (mg)
Albumin (BSA)	400 mg
Fructose	600 mg
PBS 1x	5000 mg
*Freeze-dried for 1x24 h and heated in the oven at 50°C for 60, 90, and 120 minutes	
Glycosylatedalbumin	4 mg
GSE	2 mg
NaCl	2 mL
PBS 1x	Ad to 5 mL

Additionally, GSE was dissolved in ethanol and mixed, after which the solution was freeze-dried, as noted by Huang, *et al*. (2018).

Evaluation of Albumin Glycosylation

The glycosylation index evaluation was conducted using the Bradford test. A dilution series of non-glycosylated BSA was prepared, ranging from 1000–200 µg/mL, using distilled water. The GN-GSE samples were weighed and subsequently dissolved in distilled water. A total of 10 µL of the BSA dilution series and the samples were placed into a 96-well plate. Additionally, 150 µL of Pierce reagent was added and incubated for 15 minutes. The absorbance was then measured with a plate reader (λ566 nm), and the amounts of

glycosylation albumin was calculated. Furthermore, the quality parameters of the preparation included particle size analysis (PSA), viscosity testing, and pH stability assessment.

RESULTS

Genitri Seed Extraction (GSE)

Genitri seed samples (*Elaeocarpus ganitrus*), family Elaeocarpaceae, were obtained from a genitri plant cultivation house in Kebumen. Extraction was carried out using the ultrasonic-assisted extraction (UAE) method at 40°C for 45 minutes. This method implies efficient extraction of alkaloid. The duration was considered enough to exerts the pores within hard-shield seeds (Aguilar-Hernández, *et al*., 2020). A total of 2 g of genitri seeds were extracted with 20 mL of methanol solvent (solid:liquid 1:10), the macerate solvent was evaporated and the crude extract obtained was 110 mg with yield of 5.5% (w/w). The extract obtained was fractionated with dichloromethane. After evaporation of the solvent, the fraction weight obtained was 25 mg.

Phytochemical Profiling of Genitri Seed Extract (GSE) Using Thin Layer Chromatography (TLC)

A total of 5 µL of GSE-crude extract (Cr) and GSE-fractioned extract (Fr) (20,000 ppm) was spotted on a silica 60 plate and eluted with a mobile phase of hexane: ethyl acetate: acetic acid (55%: 25%: 20%). The elution results were then dipped in Dragendroff's reagent to detect alkaloid compounds, sitroboric reagent to detect flavonoid compounds, and Anisaldehyde-H_2SO_4 reagent to detect terpenoid compounds. After heating, alkaloid compounds were marked with brown spots with Rf 0.46 in visible light. Flavonoid compounds were marked with fluorescent spots with Rf 0.31; 0.51; 0.63; and 0.73 in UV366. Meanwhile, terpenoid compounds are marked with spots with Rf 0.57 in visible light.

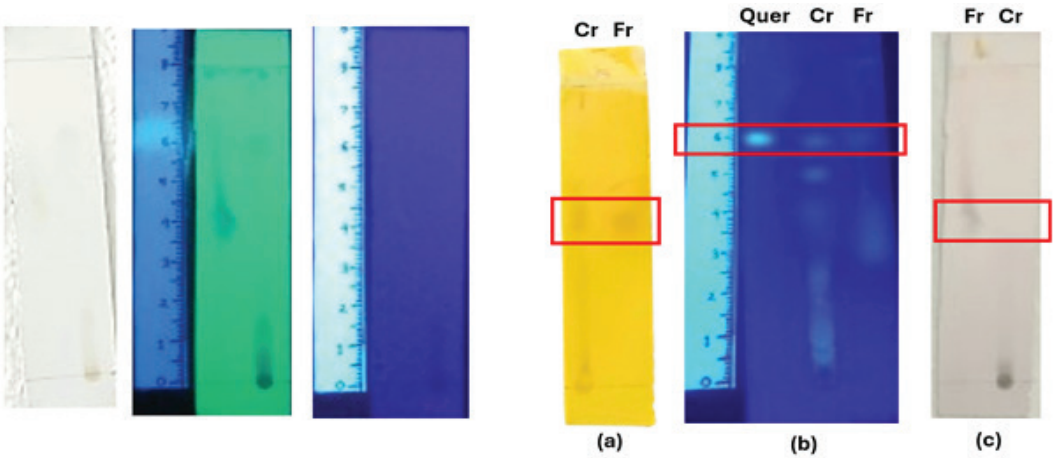

Figure 1. TLC profile of GSE after the addition of dragendorff reagent indicated the amount of alkaloid compounds (a); Sitroboric reagent reagent indicated the amount of flavonoid compounds (b); and Anisaldehyde -H_2SO_4 indicated the amount of terpenoid compounds (c).

Cytotoxic Effects of GSE on MCF-7/HER2 and Vero Cells

Cytotoxic effects indicate the toxicity of GSE (fractioned extract) to cells based on the value IC_{50}. MCF-7/HER2 cells represent cancer cells and Vero cells represent normal cells. After GSE treatment, it was found that there was a decrease in cell density and changes in cell morphology. The viability profile of MCF7/HER2 cells due to GSE administration showed an IC_{50} of

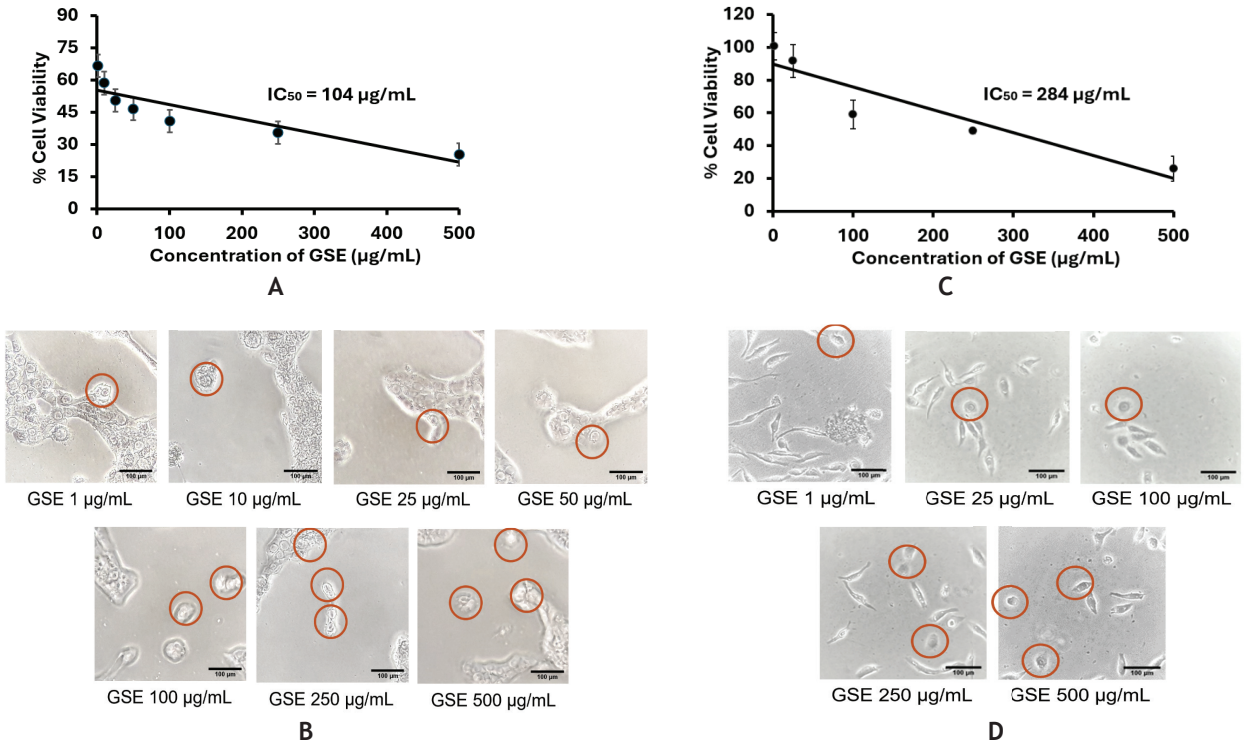

Figure 2. Cytotoxic effect of GSE on MCF-7/HER2+ cancer cell line and Vero normal cell line. A. Cell viability profile of MCF-7/HER2+, the obtained IC_{50} 104 µg/mL (R=0.851); B. Microscopy morphological of MCF-7/HER2+ treated GSE (magnification 100x); C. Cell viability profile of Vero, the obtained IC_{50} 284 µg/mL (R=0.931); D. Microscopy morphological of Vero treated GSE (magnification 100x).

104 µg/mL. Meanwhile, the viability profile of Vero cells due to GSE administration showed an IC_{50} of 284 µg/mL. The selectivity index (SI) obtained revealed the value of 2.7, indicating that GSE was selective for cancer cells.

Glycosylated-Albumin Formulation

The Glycosylated-BSA complex formation relies on the Maillard reaction, a non-enzymatic process where free amino groups in proteins interact with reducing sugars to create cross-link bonds. In this study, bovine serum albumin (BSA) and fructose were combined and dissolved in phosphate-buffered saline (PBS), followed by vortexing and freeze-drying. The resulting freeze-dried samples were then subjected to heating at 50°C for varying durations of 60, 90, and 120 minutes. The success of glycosylation was assessed by measuring free amino acid levels using the Bradford Protein Assay. Results showed that after 120 minutes, the free amino acid level decreased to 29.29%, indicating a significant extent of glycosylation.

Glycosylated-Nanoalbumin Genitri Seed Extract (GN-GSE) Formulation and Preparation Quality Control Test

GN-GSE formulation via dispersion BSA and fructose were freeze-dried to increase nanoparticle stability and increase the shelf-life of the formulation (Fonte, 2016). The preparation has physical properties of clear yellow, odorless, and a viscosity value of 10 cPs indicating that this formula is in the medium viscosity range (Berteau, *et al.*, 2015). The stability of the preparation for 3 weeks at 2°C storage showed a pH stability of 7.0.

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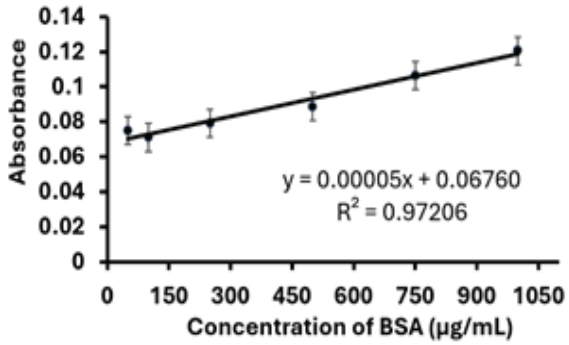
Evaluation of the preparation particles was carried out to confirm the characteristics of the nanoparticles. Based on the PSA characterization, GN-GSE has a particle size of 75.46 nm according to the size of nanoparticles. Nano sized of 10-1000 nm becomes one of factors that influences effectiveness of drug absorption. GN-GSE preparation has met the requirements of a homogeneous nano preparation with a PdI value of 0.42 (PdI<0.50) (Veronika, 2015).



A

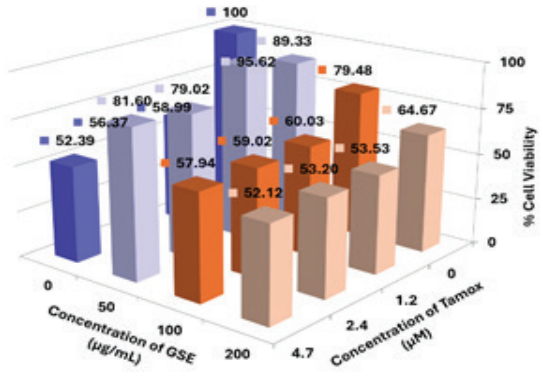
Duration (mins)	%Free Amino Acids
60	57.87% ± 0.61
90	37.00% ± 0.50
120	29.29% ± 0.20

B

Figure 3. Glycosylation Index of BSA. A. Standard curve of BSA concentrations obtained by regression, y=0.00005x + 0.00676; B. The level of free amino acids after heating process represents the amount of albumin glycosylation index.

Cytotoxic Combination Effects of Glycosylated-Nanoalbumin Genitri Seed Extract (GN-GSE) and Chemotherapy Drugs

GN-GSE formulation was obtained by glycosylation of BSA and fructose, which entrapped GSE, and act as its carrier. A cytotoxicity combination was conducted to evaluate the effects of GN-GSE on the cytotoxic effects of tamoxifen in MCF7/HER2+ cells. The results suggest that a synergistic combination of GN-GSE and tamoxifen can enhance the efficacy of tamoxifen, enabling a reduction in the treatment dose while still achieving the same level of cytotoxic effect (Muna and Jenie, 2018). Specifically, a combination of 100 µg/mL of GN-GSE with varying concentrations of tamoxifen showed a synergistic effect, indicated by a



A

Combination Index			
Concentration	Tamoxifen		
GSE	1.2 µM	2.4 µM	4.7 µM
50 µg/mL	3.16	1.28	2.40
100 µg/mL	0.55	0.69	0.96
200 µg/mL	0.76	0.89	1.12

B

Figure 4. Cytotoxic combination of GN-GSE and Tamoxifen. A. Cell viability profile of MCF-7/HER2+ treated GN-GSE (containing GSE 50; 100; 200 µg/mL) and Tamoxifen (1.2; 2.4; 4.7 µM); B. Combination Index (CI) value of GSE-Tamoxifen, CI <1.0 determined as synergistic combination.

Combination Index <1.0 (Haagensen, *et al*., 2012). Overall, the combination of GN-GSE with tamoxifen was found to inhibit cell growth more effectively than either treatment alone at the same concentration.

Molecular Docking of Apoptosis Regulator Proteins With MOE 2010

The affinity interaction of GSE compounds-induced apoptosis was conducted through molecular docking on the CDK1 target protein. Target protein selection was based on data from the UALCAN database (https://ualcan.path.uab.edu/analysis.html), which identifies overexpressed genes in HER2+ breast cancer, and Swiss Target Prediction (http://swisstargetprediction.ch/), which indicates proteins targeted by specific compounds. Results were evaluated using InteractiVenn to analyze overlaps (Chandrashekar, *et al*., 2022). CDK1 (Cyclin-Dependent Kinase 1) regulates the G2/M phase of the cell cycle. The docking results indicated that the main GSE compounds had a stronger affinity for CDK1 than the native ligand, Dinaciclib, a known inhibitor. A more negative docking score (ΔG) signifies better affinity and inhibition. Consequently, three GSE compounds were identified as effective CDK1 inhibitors, as shown in Table 2 and Figure 5.

Tabel 2. *S-score docking* senyawa utama GSE.

Ligand	Native ligand*	Elaeocarpenine	Elaeocarpucine C	Quercetin
S-score	-5.80 kcal/mol	-11.40 kcal/mol	-13.71 kcal/mol	-13.92 kcal/mol

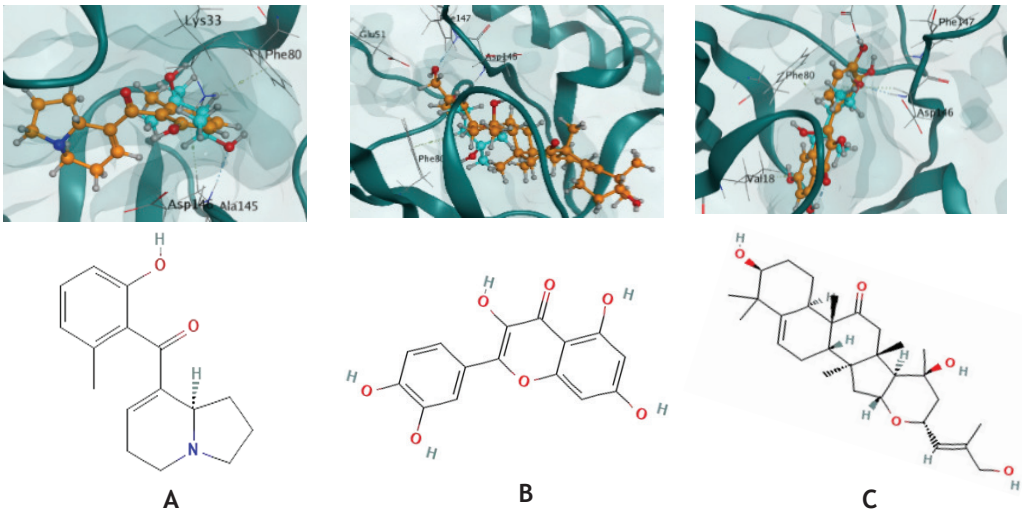


Figure 5. Visualization of ligand interaction on CDK1. The native ligand shown in blue-colored, while the target compounds shown in orange-colored: A. Elaeocarpenine; B. Quercetin; C. Elaeocarpucine C.

DISCUSSION

This research aims to explore the cytotoxic effects of Genitri Seed Extract (GSE) as a co-chemotherapy agent that induces apoptosis in HER2-overexpressing breast cancer formulated in glycosylated drug delivery nanoalbumin. Genitri plants (*Elaeocarpus ganitrus*) are known to have anticancer effects with moderate cytotoxicity against BxPC-3 and HeLa cell lines (Utami, *et al*., 2013). The anticancer activity of genitri plants is supported by flavonoid, terpenoid, and alkaloid compounds, with a typical compound, namely elaeocarpenine, which is found in large quantities in the seeds

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(Yogesh, *et al*., 2017). Extraction of compounds carried out with methanol to dissolve polar and nonpolar analytes in GSE, one of which is alkaloids. Alkaloid fractionation is carried out by dichloromethane due to its volatile nature and the presence of chlorine groups can facilitates the separation of compounds and protects compounds from damage due to high temperatures (Emilda, *et al*., 2023).

Phytochemical profiling of GSE was performed using TLC with visualization reagents to identify the presence of compounds. Dragendorff's reagent detected alkaloids, yielding a reddish-orange color (Chairunnisa, *et al*., 2019). The Sitroboric reagent was used to identify flavonoids, it reacts with the ortho-hydroxy group yielding a bright yellow color that fluorescence under UV254 visualization (Hikmawanti, 2023), while anisaldehyde-H_2SO_4 revealed terpenoids with a purple color (Masadi, *et al*., 2018). The analysis found alkaloids at Rf of 0.47, flavonoids at Rf 0.73, and terpenoids at Rf 0.57, which linked to anticancer effects.

The anticancer effects of GSE were studied using the MCF-7/HER2+ breast cancer cell model, which overexpresses the HER2 receptor. This receptor activates proliferation and anti-apoptotic signals through the PI3K pathway, Bcl-2, and IAP proteins, inhibiting apoptosis (Siddiqa, *et al*., 2008). The safety of GSE was also tested on Vero cells, an African green monkey kidney epithelial line. The IC_{50} value for MCF-7/HER2+ cells was 104 µg/mL, while for Vero cells, it was 284 µg/mL, yielding a selectivity index (SI) of 2.7, indicating a preference for cancer cells over normal cells (Artun *et al*., 2016). Combination of GSE with the chemotherapy agent, tamoxifen, showed a synergistic effect with CI value of less than 1.0 at most concentrations (Ho, *et al*., 2021). This synergy may enhance therapy effectiveness, reduce drug resistance, and lower side effects (Ho, *et al*., 2021).

HER2 mutations in HER2+ type breast cancer cause chemotherapy resistance and increased dimerization of the HER2 family (Ron, *et al*., 2021). The application of glycosylated drug delivery nano-albumin helps overcome the mechanism of cancer

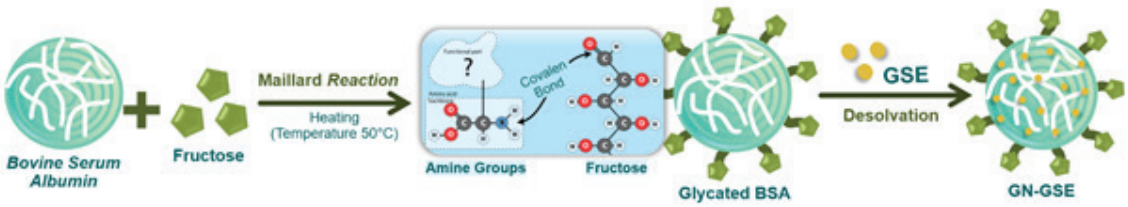

Figure 6. Albumin glycosylation proposed mechanism.

drug resistance by specifically targeting cancer cells utilizing the principle of glycosylation of glucose derivatives of aerobic glycolysis as a characteristic of cancer cells (Koppenol, *et al*., 2011). Protein nanocarriers, especially albumin based nanoparticles, offer various benefits compared to other nanomaterials. Biocompatibility, biodegradability, and low toxicity are the characteristics of protein nanocarriers (Verma, 2018).

Glycation is based on the *Maillard reaction*, which is the process of conjugating proteins with reducing sugars through covalent bonds upon heating which forms glycated protein products (Asyhari, 2018). Glycated success parameters based on the Bradford Protein Assay. The principle of this method involves binding protein molecules with coomassie blue in acidic conditions which causes a color change from brown to blue. A decrease in albumin concentration correlates with a decrease in % free amino which indicates that the α-amino group of tripeptide has been bound to the carbonyl group of sugar (Suseno, 2016). The best result obtained the lowest free amino of 29.29% at a

temperature of 50°C for 120 minutes. The appropriate heating duration can catalyze the Maillard reaction which faster the glycosylation process (Asyhari, 2018).

According to Primiani, *et al*. (2021), the methanolic extract of GSE contains unique phytochemical compounds from the *Elaeocarpus* family, including 1.88% Elaeocarpenine, 1.85% Elaeocarpucine C, and high amount of Quercetin 2.51%. These compounds represent the major constituents of alkaloids, terpenoids, and flavonoids. The presence of these 3 classes of compounds was confirmed by TLC, indicating that Elaeocarpenine, Elaeocarpucine C, and Quercetin were present as it were the major components.

GN-GSE selectively accumulates in cancer cells through glucose transporters overexpressed on the cell membrane. GLUT-5 is responsible for fructose uptake and is overexpressed in MCF-7 cell line in the breast cancer model (Zhou, *et al*., 2017). In MCF-7/HER2+ cells, increased proliferation occurs due to the binding of the CDK1 complex to *cyclin B1* in the G2-M phase. HER2 dimerization activates the PI3K pathway and ERK pathways which causes a decrease in p21 expression as a CDK1 inhibitor. The decrease in CDK1 inhibitors makes the activation of the proliferation pathway uninhibited (Saatci, *et al*., 2018). In this case, the affinity of the GSE compound complex as a CDK1 inhibitor is an important target for inducing apoptosis.

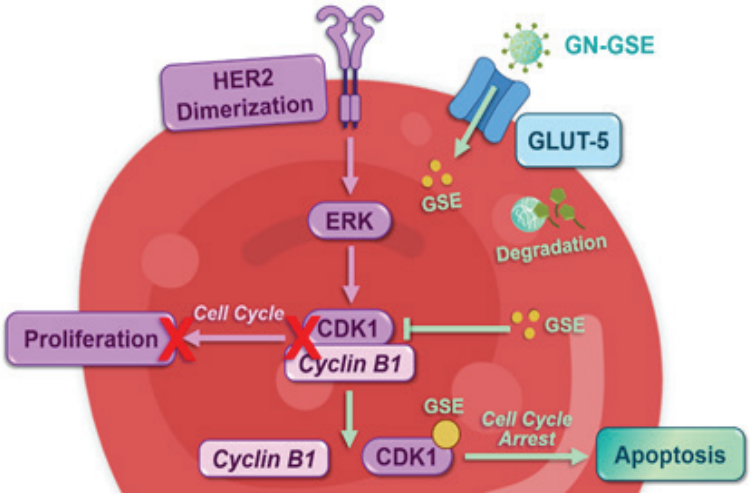

Figure 7. Proposed mechanism of apoptosis induction by disruption of cyclin-CDK1 complex.

The bioinformatics study using molecular docking demonstrated that these three major compounds from GSE exhibit strong binding affinity to CDK1, with the following values: Quercetin at -13.92 kcal/mol; Elaeocarpucine C at -13.71 kcal/mol; Elaeocarpenine at -11.40 kcal/mol in comparison with native ligand Dinaciclib at -5.80 kcal/mol. Although Elaeocarpenine was the unique compound of GSE, Quercetin has higher affinity among the rest of two major compounds, followed by Elaeocarpucine C, than Elaeocarpenine. The presence of these 3 major compounds suggests that GSE may alter the interaction between CDK1 and cyclin B way better, thereby preventing its activation and influencing cell cycle arrest on G2/M-phase. The more negative ΔG value, the stronger the binding affinity and the more effective on inhibition of CDK1 (Ortiz, *et al*., 2019). The binding of Elaeocarpenine, Elaeocarpucine C, and Quercetin to CDK1 prevents CDK1 from

associating with cyclin B1, leading to cell cycle arrest. This mechanism is what induces apoptosis in breast cancer cells (Wijnen, *et al*., 2021).

CONCLUSION

GSE contains alkaloids, flavonoids, and terpenoids that exhibit anticancer properties. GSE specifically targets cancer cells, such as MCF-7/HER2+, while being non-toxic to normal cells like Vero. The apoptosis-induced effect by GSE occurs through the inhibition of CDK1, a mechanism that shows a strong affinity, as indicated by a higher s-score value of the target compound compared to the native ligand. Additionally, the glycosylated drug delivery formulation of GN-GSE demonstrates good stability over a storage period of three weeks and meets the criteria for nanoparticles.

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