Trop J Nat Prod Res, January 2025; 9(1): 67 - 72

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Enhancing Chemotherapeutic Efficacy: Synergistic Cytotoxic Effect of *Garcinia cowa* Bark Extract and Doxorubicin in T47D Breast Cancer Cells

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ARTICLE INFO

Article history:
Received 22 October 2024
Revised 23 November 2024
Accepted 10 December 2024
Published online 01 February 2025

ABSTRACT

Breast cancer remains one of the leading causes of mortality worldwide, demanding more effective and less toxic treatment strategies. The investigation of natural compounds as potential adjuvants to chemotherapy has gained urgency in recent years. The aim of this research was to investigate the cytotoxic effects of *Garcinia cowa* bark ethanol extract (GCBEE) alone and its potential to enhance the cytotoxic activity of doxorubicin on T47D breast cancer cells, as well as to assess the selectivity of GCBEE towards the Vero cell line. The MTT assay was utilized to evaluate the cytotoxic effects of doxorubicin (dox) and GCBEE on T47D breast cancer cells. Selectivity against normal Vero cell lines was evaluated using the selectivity index (SI). The test results were examined using a microplate reader set to operate at 595 nm wavelength. Chou Talalay's method was utilized to compute and analyze the combination index (CI) in order to ascertain the influence of the combination. Based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, both GCBEE and dox exhibited concentration-dependent cytotoxic effects with IC_{50} values of 130 µg/mL and 0.026 µg/mL on T47D cells, respectively. GCBEE was more selective for T47D cells than Vero cells (SI = 15). GCBEE synergistically enhanced the cytotoxic effect of dox, with a CI value range of 0.19–0.89. The combination of GCBEE and doxorubicin offers a promising synergistic approach in the fight against breast cancer.

Keywords: Cytotoxic activity, *Garcinia cowa*, Combination index, Breast cancer, Co-Chemotherapy.

Introduction

Breast cancer continues to be a major global health issue for women and a leading cause of mortality.[1] Every year, cancer-related morbidity and mortality continue to increase. This has been complicated with the cost of chemotherapy and its devastating side-effects.[2] The development of multidrug resistance pathways in cancer cells frequently reduces the efficacy of chemotherapeutic medicines like doxorubicin.[3] Long-term use of doxorubicin has also been reported to cause cardiotoxicity.[4] Despite advancements in treatment, current breast cancer therapies often lack selectivity, leading to significant damage to normal cells and limiting their effectiveness.[5] As such, It is crucial to develop new cancer treatments that can specifically target and eliminate cancer cells while protecting healthy tissues, thereby improving therapeutic outcomes and reducing adverse side effects.[6] Thus, one of the main objectives of contemporary scientific study is to find many ways to maximise the effectiveness of doxorubicin in cancer cells and reduce the related toxicity in non-cancerous tissues. [7]

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Citation: Ifora I, Hamidi D, Susanti M, Hefni D, Wahyuni FS. Enhancing Chemotherapeutic Efficacy: Synergistic Cytotoxic Effect of *Garcinia cowa* Bark Extract and Doxorubicin in T47D Breast Cancer Cells. Trop J Nat Prod Res. 2025; 9(1): 67 – 72 https://doi.org/10.26538/tjnpr/v9i1.10

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The combination of doxorubicin with chemopreventive agents is needed to increase doxorubicin activity, overcome drug resistance, and reduce side effects.[8] Developing novel treatment strategies for breast cancer continues to be one of the most challenging fields in cancer research.[9] Numerous tropical plants have biological properties that may have medical uses.[10] The family *Guttiferae* includes *Garcinia cowa* Roxb, also referred to as "kandis," which is found in tropical rainforests in countries like Thailand, Indonesia, Malaysia, and the Philippines.[11] Traditional medicine has utilised several parts of *Garcinia cowa*. Bark, latex, leaves, and roots in the management of burns, wounds, pain, and inflammation.[12] The chemical composition and biological activity of various parts of *Garcinia cowa* have been studied previously.[13] *Garcinia cowa* contains various active compounds, including *cowanin*, *rubraxanthone*, *cowaxanthone*, and *norcowanin*.[13–16] Several studies have consistently reported that a variety of plant extracts possess anticancer activity, including the ability to suppress the growth of T47D breast cancer cells.[17–20] Xanthone isolated from methanol extract of bark can suppress the growth of MCF-7 cells.[21] This demonstrates that the *Garcinia cowa* could serve as a lead in the search for anticancer compounds. This study was conducted to investigate the cytotoxic effects of an ethanolic extract of *Garcinia cowa* Bark in combination with doxorubicin on T47D breast cancer cells.

Materials and Methods

Plant material

The bark of *Garcinia cowa* Roxb. was collected in December 2022 at Kudu Gantiang, Pariaman City, West Sumatra. The plant material has been deposited at the Herbarium of Andalas University, West Sumatra,

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Indonesia, under the voucher number 556-ID/ANDA/XII/2022. Dr. Nurainsa, a botanist from Andalas University's Herbarium, identified the *Garcinia cowa* Roxb. bark.

Preparation of plant material and extraction
Plant materials were sliced into tiny pieces (3-5 mm thick) and allowed to air-dry in a shaded area for 7 days. The dried bark of *Garcinia cowa* was ground into a powder using a traditional grinder. The materials were then soaked for 24 hours at room temperature in 70% ethanol with intermittent stirring and then filtered. This process was repeated thrice. The filtrates were combined and concentrated under a vacuum using a rotary evaporator at 45℃ till a brownish semisolid extract was formed. The extract was kept in a refrigerator at 4℃ for further pharmacological testing.

Cell culturing procedure
T47D cells, a kind of human breast cancer, were provided for the study by Prof. Masashi Kawaichi of the Nara Institute of Science and Technology in Japan. Vero cells were collected from the Gajah Mada University. T47D and Vero cells were grown in Dulbecco's modified Eagles medium, which contained 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin-1% streptomycin (Gibco, Grand Island, NY, USA), and 0.5% fungizon (Gibco, Grand Island, NY, USA), in a flask in a humidified atmosphere (5% CO_2) at 37˚C.[22]

Single Cytotoxic assay
T47D cells were seeded in 96-well plate with $1x10^4$ cells/well and divided into control and treatment group. Then, incubated in 37˚C with 5% CO_2 for 24 hours. Cells were treated with final concentrations of GCBEE were 18.75, 37.5, 75, 150, and 300 µg/mL while the concentrations of doxorubicin were 0.001, 0.01, 0.1, 1 and 10 µg/mL. The culture media was withdrawn and the cells were cleaned with PBS (Sigma) after a 48-hour incubation period. 100 µL of diluted 5 mg/mL MTT on PBS (Sigma) was applied to each well after being diluted with Dulbecco's Modified Eagle Medium (DMEM). After four hours of incubation, 10% sodium dodecyl sulfate (SDS) in HCL 0.01 N was added to stop the reaction. After that, the plate was incubated for one night at room temperature in a dark environment. The plate was agitated for ten minutes to ensure that the formazan had dissolved, and then the absorbance was measured at a wavelength of 595 nm using an ELISA reader (Bio-Rad, USA). Every treatment was administered in triplicate, and the cytotoxic activity was quantified using the IC_{50} method, which determines the quantity needed to lower the population's absorbance of cells by 50% in comparison to the untreated (control) cells.

Combination Cytotoxic Assay
The combination test was carried out using the same method after determining the IC_{50} value of a single treatment. In the combination test, the concentrations of GCBEE were 520, 260, 130, 65, and 32 µg/mL, while the concentrations of dox were 0.104, 0.052, 0.026, 0.013, and 0.007 µg/mL, corresponding to 4, 2, 1, ½, and ¼ of their respective IC_{50} values. Next, the Combination Index (CI) was calculated. Based on the Chou-Talalay method, CompuSyn software calculates CI values that show the impact of medication combinations.[23]

Selectivity index
To determine the cytotoxic selectivity of GCBEE, the Selectivity Index (SI) was calculated according to the following equation **(1)**.[25]

$$SI = \frac{\text{IC50 on Normal Cells}}{\text{IC50 on Cancer Cells}} \quad \textbf{(1)}$$

Extract is non-selective if SI is less than 3, but selective if SI is greater than 3.[26]

Statistical analysis
The *in vitro* experiment data were presented as mean ± S.E.M. Using the obtained absorbance, the percentage (%) of cell viability was determined. Graphical illustration of the viability percentage and IC_{50} value data were analyzed and presented using GraphPad Prism (GraphPad Prism, 9.0.0). The effects of medication combinations are displayed by CI values calculated using CompuSyn software (Vers.1.0).

Results and Discussion

Single Cytotoxic assay and Selectivity index
Cytotoxicity is categorized by the National Cancer Institute (NCI) based on IC_{50} values. Extracts with IC_{50} values less than 20 µg/mL are considered highly active, moderately active if IC_{50} 21-200 µg/mL, weakly active if IC_{50} = 201–500 µg/ml and IC_{50} values above 500 µg/mL indicate inactive or no cytotoxicity.[27] The inhibitory concentration (IC_{50}) is the amount needed to prevent 50% of cell growth. Lower IC_{50} values signify larger efficacy in inhibiting cell division.[28,29]

Table 1: Interpretation of CI value.[24]

CI	Interprestation	CI	Interprestation
< 0.1	Very strongly synergistic	0.90-1.10	Nearly additive
0.1-0.3	Strongly synergistic	1.10-1.20	Slight antagonist
0.3-0.7	Synerggistic	1.20-1.45	Middle antagonist
0.7-0.85	Moderate synergistic	1.45-3.3	Antagonist
0.85-0.90	Slight synergistic	>3.3	Strongly antagonist

The GCBEE exhibited an IC_{50} of 130 µg/mL on T47D cells, indicating moderate cytotoxic activity. In contrast, dox showed a significantly lower IC_{50} of 0.026 µg/mL, highlighting its potent cytotoxicity as a chemotherapeutic agent. GCBEE's IC_{50} on Vero cells was considerably higher, at 1.955 µg/mL, suggesting a lower toxic effect on non-cancerous cells. The selectivity index (SI) is a crucial metric to evaluate the specificity of GCBEE towards cancer cells relative to non-cancerous cells. A higher SI indicates better selectivity and potential as an anti-cancer agent.[30] For GCBEE, the SI was 15 for T47D cells versus Vero cells (Table 2). An SI > 3 is considered to exhibit high selectivity, underscoring GCBEE's promising anti-cancer potential with relatively low toxicity towards normal cells. Dox did not have an SI calculation in this study as the IC_{50} on Vero cells was not determined, but it is known to have significant toxicity in both cancer and normal cells.[31] The results of this study demonstrate that *Garcinia cowa* bark ethanol extract (GCBEE) possesses selective cytotoxic activity against T47D breast cancer cells, as evidenced by an IC_{50} value of 130 µg/mL and a high selectivity index (SI = 15) when compared to Vero cells. The SI value exceeding 3 suggests that GCBEE has a strong potential as an anti-cancer agent with reduced toxicity towards normal, non-cancerous cells.[26] This high selectivity makes GCBEE a promising candidate for further investigation in cancer therapy, especially as a natural compound with possibly fewer side effects than conventional chemotherapeutic agents like doxorubicin.

Table 2: IC_{50} of GCBEE and dox on T47D cells, Vero cells and SI[a] values of GCBEE at 48 h after administration.

	Cell Lines	
IC_{50} (µg/mL)	T47D	Vero
GCBEE	130	1.955
Dox	0.026	-
[a]SI		
GCBEE	15	1

[a]SI : refers to Selectivity Index.SI values > 3 was considered as high selectivity.
- : IC_{50} was not calculated.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Although GCBEE's IC_{50} value is higher than that of doxorubicin (0.026 µg/mL) (Figure 1), its significantly lower cytotoxicity towards Vero cells offers a considerable advantage in reducing side effects. Doxorubicin, while effective at killing cancer cells, is known for its severe side effects due to its non-selective nature, affecting both cancerous and normal cells. The moderate cytotoxicity of GCBEE towards cancer cells combined with its minimal impact on normal cells supports its potential as a safer alternative or complementary therapy in breast cancer treatment. Future research could further explore the bioactive compounds within GCBEE responsible for the cytotoxic activity. Additionally, *in vivo* studies are needed to confirm the therapeutic potential and safety of GCBEE in a biological system. The development of targeted delivery systems could further enhance its efficacy, reduce required dosages, and mitigate any residual toxicity towards normal cells. This study provides a solid foundation for the continued exploration of GCBEE as a natural anti-cancer agent, offering a balance between efficacy and safety that is critical for developing new cancer therapies.

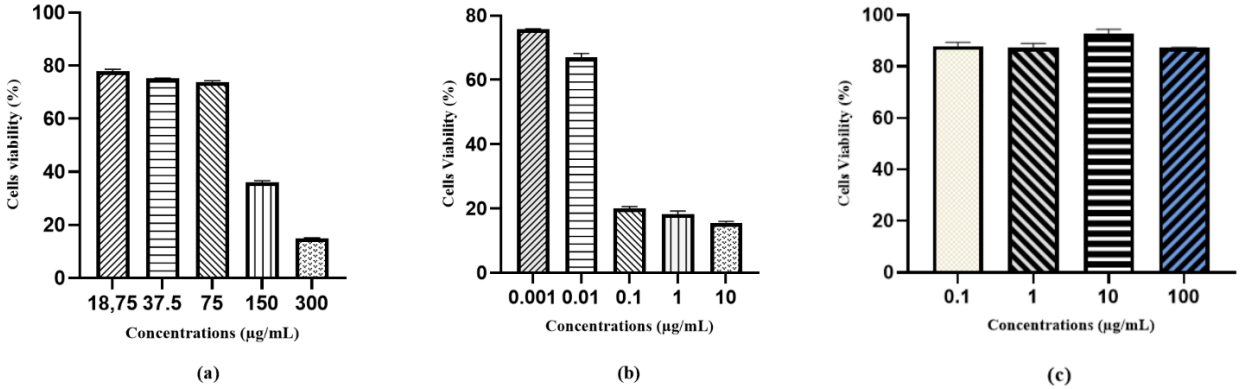

Figure 1: Cell viability (%) profile of T47D cells following treatment with GCBEE (IC_{50}= 130 µg/mL) (a), doxorubicin (IC_{50}= 0.026 µg/mL) (b), and Cell survivability (%) of Vero cells following treatment with GCBEE (IC_{50}= 1.955 µg/mL) (c) for 48 h single test application. Viability values are presented as mean (n = 3) ± SEM.

Combination Cytotoxic Assay

The cytotoxic effects of GCBEE on T47D cells are concentration-dependent (Figure 2a). As the concentration increases from 32.5 µg/mL to 520 µg/mL, cell viability decreases significantly. The viability of the cells decreases significantly at the maximum dose of 520 µg/mL, suggesting that GCBEE is an effective means of lowering cell viability on its own. Figure 2b shows the cytotoxic effects of dox on T47D cells. Similar to GCBEE, dox exhibits a concentration-dependent reduction in cell viability. At lower concentrations (0.007 µg/mL), the impact on cell viability is minimal. However, as the concentration rises to 0.104 µg/mL, there is a substantial reduction in cell viability, indicating dox's well-known efficacy as a chemotherapeutic agent. These findings are consistent with dox's mechanism of action, which involves DNA intercalation and inhibition of topoisomerase II, resulting in the death of rapidly proliferating cells, like cancer cells.[32] This validates dox's potency against breast cancer cells in a controlled in vitro environment.

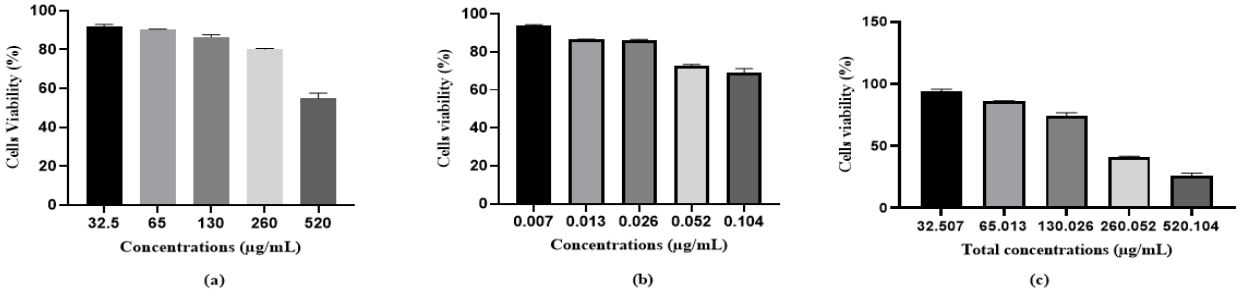

Figure 2: Cell viability (%) profile of GCBEE (a), dox (b), and combination GCBEE + dox (c) on T47D cells in the combination test application. All values are expressed as means (n = 3) ± SEM.

The combined treatment of GCBEE and dox demonstrates an even greater reduction in cell viability compared to each compound alone, particularly at higher concentrations. At 520.104 µg/mL (Figure 2c), the combination achieves a near-complete reduction in cell viability. The combination effect is likely synergistic, as indicated by a greater-than-expected reduction in cell viability, which suggests that GCBEE may enhance the cytotoxic effects of dox on T47D cells. This could be due to complementary mechanisms of action between GCBEE and dox. These findings support the potential of GCBEE as an adjuvant to traditional chemotherapy, especially to increase dox's effectiveness in the treatment of breast cancer. Further studies are necessary to explore the exact molecular interactions and to determine if similar effects can be achieved in vivo.

The most common usage of drug combinations is to treat the chronic diseases, like AIDS and cancer. The major goals are to reduce dosage and toxicity, produce a synergistic therapeutic effect, and prevent the onset of drug resistance.[33] The quantitative definitions of synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1) as shown in Table 1 , as well as the techniques for their computer simulation, are obtained by combining the median-effect equation with the CI equation.[24,34] Based on the Chou and Talalay approach, the CI was computed to

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

ascertain if the combination of GCBEE and dox has an antagonistic, additive, or synergistic effect.[35]

The Combination Index (CI) values for T47D breast cancer cells when *Garcinia cowa* bark ethanol extract (GCBEE) and doxorubicin were combined at different doses are shown in Table 3, as determined by the Chou-Talalay method using Compusyn software. At a total dose of 520.104 µg/mL, the combination affects 74.2% of the T47D cells (Fa = 0.742), with a CI value of 0.19, indicating a strong synergistic effect. A dose of 260.052 µg/mL affects 58.9% of the cells (Fa = 0.589) with a CI value of 0.25, suggesting a synergistic interaction. The 130.026 µg/mL dose affects 26.0% of the cells (Fa = 0.260) with a CI value of 0.89, still indicating synergism, albeit weaker compared to the higher doses. At a lower dose of 65.013 µg/mL, the fraction affected drops to 14.2% (Fa = 0.142), and the CI value increases to 1.27, suggesting antagonism. The smallest dose of 32.507 µg/mL affecteds only 6.3% of the cells (Fa = 0.063) with a CI value of 2.23, indicating strong antagonism.

Table 3: Combination Index values of combination GCBEE and doxorubicin on T47D cells

Total Dose (µg/mL)	Fraction Affected (Fa)	Combination Index (CI) Value
520.104	0.742	**0.19**
260.052	0.589	**0.25**
130.026	0.260	**0.89**
65.013	0.142	1.27
32.07	0.063	2.23

The cytotoxicity analysis of the combination of *Garcinia cowa* bark ethanol extract (GCBEE) and doxorubicin on T47D cells reveals a significant synergistic effect at higher doses, as demonstrated by CI values well below 1 at doses of 520.104 µg/mL and 260.052 µg/mL. The highest degree of synergy is observed at the dose of 520.104 µg/mL, where 74.2% of the cells were killed, and the CI value reaches an impressive 0.19, indicating potent synergy. This suggests that at higher dose combinations, the two agents work in concert to exert enhanced cytotoxic effects, likely reducing the required concentration of doxorubicin to achieve similar levels of cytotoxicity, thereby potentially minimizing its toxic side effects.

The dose-effect curve Figure 3a clearly demonstrates that doxorubicin (dox) alone (red curve) has a potent cytotoxic effect at very low doses, while GCBEE (green curve) requires higher doses to reach similar levels of efficacy. The combination of the two (GCBDox, blue curve) shows an improved response compared to GCBEE alone, suggesting that combining these agents enhanceds their cytotoxic effect, particularly at higher doses.

The combination index (CI) plot (Figure 3b) further supports this finding. CI values below 1 across a range of affected fractions (Fa) indicate a synergistic effect between GCBEE and dox, especially as the fraction affected increases. This synergy suggests that the combination is more effective in killing cancer cells compared to each agent used individually, particularly at doses higher than 0.2 Fa. However, as the dose decreases, the CI values progressively increase, with the combination becoming antagonistic at the lower doses of 65.013 µg/mL and 32.507 µg/mL. This shift from synergy to antagonism at lower doses may indicate that below a certain concentration, the interaction between GCBEE and dox becomes antagonistic, possibly due to inadequate cellular exposure to either agent. This finding is also in line with the isobologram curve (Figure 3c), The combination of *Garcinia cowa* extract and doxorubicin showed a synergistic effect at Fa 0.5, 0.75, and 0.9, with the isobologram curve below the straight line, indicating that the combination dose is more effective than the single agent. Similar findings related to the synergistic properties of the combination of natural extracts and conventional cytotoxic drugs have also been reported by several researchers, such as those conducted by previous researchers who reported that synergistic effects are demonstrated when an anti-cancer medication and a natural chemical are used together, improving total therapeutic activity against cancer cells.[36]

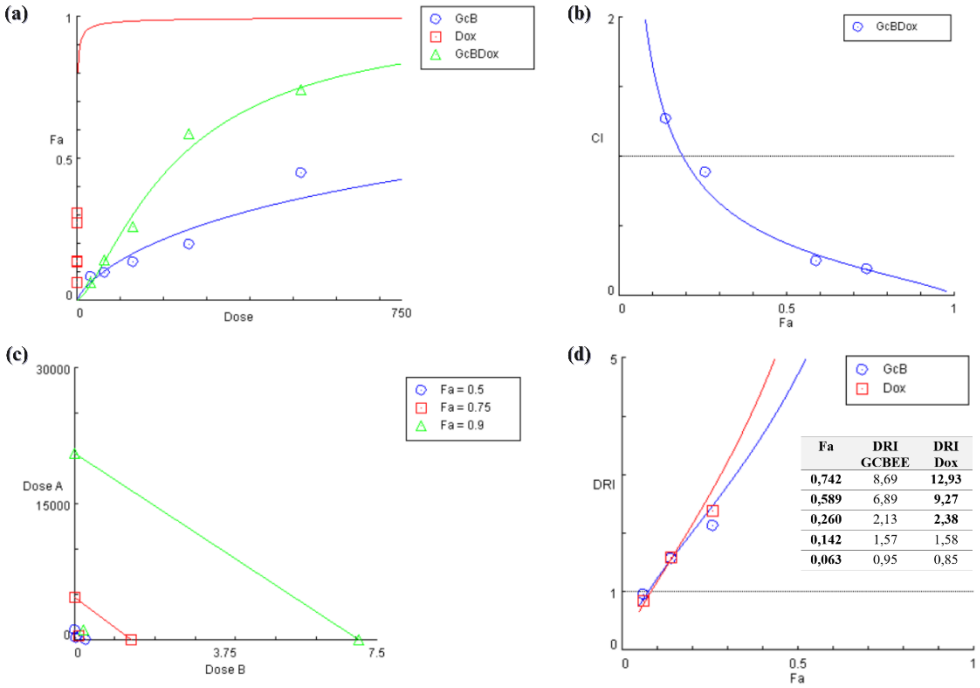

Figure 3: Concentration-effect curve (a), combination index plot (b), and isobologram (c) drug reduction index plot (d).

Additionally, other studies also reports that substances derived from plants have a significant influence as therapeutic agents, particularly cytotoxic agents, whether used alone or combined with other medications.[37]

The DRI plot and the accompanying table of DRI (Figure 3d) values provide essential insights into how much the doses of GCBEE and doxorubicin can be reduced when used in combination, without compromising efficacy.At an Fa of 0.742, the DRI for GCBEE is 8.69106 and for dox is 12.8295, indicating that the simultaneous use of both drugs significantly reduced the dosage while still achieving high levels of cell death. This reflects a considerable improvement in therapeutic potential, especially for doxorubicin, whose dose reduction could help alleviate its known toxic side effects, such as cardiotoxicity. As the Fa decreases, the DRI values decrease as well, suggesting that dose reduction is less prominent at lower fractions affected. However, even at lower Fa (e.g., 0.142), the DRI values remain above 1 for both agents, reinforcing the idea that dose reduction is still possible across a broad range of therapeutic effects. GCBEE shows consistent dose reduction across various Fa levels, implying that this natural extract, when combined with doxorubicin, can enhance the overall therapeutic response and mitigate the need for higher doses of the chemotherapeutic agent. The DRI data suggest that combining GCBEE with doxorubicin can lead to synergistic cytotoxicity against T47D breast cancer cells. This synergism allows for lower doses of both agents to achieve similar therapeutic effects, which is particularly significant for doxorubicin due to its well-documented toxicity profile. Reducing the dose of doxorubicin while maintaining its anticancer efficacy offers a significant clinical advantage, as it could minimize adverse effects, improve patient tolerance, and enhance the quality of life for those undergoing chemotherapy.

Conclusion

These results strongly indicate that *Garcinia cowa* Bark ethanolic extract displayed higher selectivity for T47D cells over Vero cells and synergistically enhanced cytotoxic activity of doxorubicin. These findings suggest the potential of GCBEE as a selective and synergistic adjunct to dox in cancer therapy. The results of this study open avenues for further preclinical and clinical investigations to explore the full therapeutic potential of this combination. Future studies should aim to confirm these synergistic effects *in vivo* and elucidate the underlying molecular mechanisms behind the observed synergy, thus paving the way for developing more effective and safer combination therapies for breast cancer treatment.

Conflict of Interest

The authors declare no conflicts of interest.

Author's Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The research was funded by the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia through the Basic Research scheme of the Doctoral Dissertation Research cluster with contract No. 041/E5/PG.02.00.PL/2024 subcontract No. 14/UN16.19/PT.01.03/PL/2024, for which the authors are grateful. We want to express our sincere gratitude to Prof. Masashi Kawaichi of the Nara Institute of Science and Technology, Japan, for providing the T47D breast cancer cells for this study, with special assistance from Gajah Mada University.

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