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In-vitro synergistic cytotoxicity effect of *Eurycoma longifolia* herbal infusion with Tamoxifen drug against breast cancer cell line MDA-MB-231

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Abstract:

Breast cancer is considered as one of the most common diagnosed cases among prevalent malignancy cancer. This study explores the synergistic interactions between *Eurycoma longifolia* Jack herbal infusion (ELH) and tamoxifen in inhibiting breast cancer cell viability. Methodologically, *E. longifolia* leaves were collected, steam-blanched, ground, and freeze-dried. Herbal infusion, replicating common brewing conditions, was prepared using hot water extraction. The MDA-MB-231 human breast cancer cell line was employed for combinatorial MTT assays, assessing cell viability with various concentrations of extract and tamoxifen. Combination indices were calculated to determine the degree of synergy. The finding presented through combination indices, suggest varying degrees of synergy between the extract and tamoxifen, indicating enhanced therapeutic efficacy. The lowest percentage of MDA-MB-231 cell viability was shown by the combination of 2.5 µg/ml tamoxifen and 100 µg/ml ELH at 25.95%. Meanwhile, the combination of 10 *ug/ml* tamoxifen and 100 *ug/ml* of ELH displayed a strong synergistic effect of cytotoxicity towards cancer cell viability. Therefore, it is validated that the potential of ELH to exerted cytotoxicity synergistic effect with tamoxifen against the breast cancer cell.

Keywords — ***Eurycoma longifolia*, herbal infusion, synergistic, cytotoxicity, breast cancer.**

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I. INTRODUCTION

Breast cancer is a global health concern with 2.3 million new cases worldwide in 2020. It's the leading cancer for women, causing 685,000 deaths [1]. According to the latest WHO data published in 2020, breast cancer new cases in Malaysia reached 8,418 cases and mortality number of 3,503 deaths which rank second highest contributor after lung cancer [2]. The risk factors for cancer occurrence are mainly due to age, family history, genetics, hormones, and lifestyle [1].

Cancer treatment employs a multifaceted approach with various drug classes. Chemotherapy disrupts cancer cell division, targeted therapies focus on specific molecules, and immunotherapy enhances the immune system's ability to combat cancer [3]. Tamoxifen is a selective estrogen receptor modulator (SERM) used in the treatment of hormone receptor-positive breast cancer. It

functions by blocking estrogen receptors in breast cells, inhibiting the growth of estrogen-stimulated cancers. Additionally, tamoxifen is utilized for breast cancer risk reduction and as adjuvant therapy post-surgery [4],[5].

Cancer patients often explore herbal remedies alongside conventional treatments to manage symptoms and improve quality of life [6],[7]. Some medicinal plants, rich in bioactive compounds like polyphenols and flavonoids, have shown anti-cancer properties in lab studies, with potential antioxidant, anti-inflammatory, and anti-proliferative effects [8]-[11]. However, using medicinal plants for cancer treatment poses risks, including interactions with conventional treatments or medications [12]. Understanding the synergistic interaction between herbal and cancer medicine is crucial for unlocking new treatment modalities.

Eurycoma longifolia Jack. or "Tongkat Ali", is a flowering plant native to Southeast Asian countries such as Malaysia, Indonesia, Thailand, and Vietnam. It is a tall, slender shrub that can grow up to 10 meters in height. It has long history of traditional use in Southeast Asian folk medicine. It is often used to treat various ailments, including malaria, fevers, and sexual dysfunction [13]-[15]. In previous studies, the cytotoxicity effect of this plant's root had been demonstrated against many cancer cell lines, such as leukemia cell lines K-562 and HL-60 [16], human breast cancer cell line MCF-7 [17] and cervical cancer cell line HeLa [18]. However, the consumption of its leaf infusion using hot water is rare and to date, its scientific information regarding its benefit remains inadequate.

Therefore, this present study aim is to determine the potential of *E. longifolia* leaves as an herbal tea with tamoxifen drug to contribute a synergistic cytotoxic effect towards a cancer cell line, MDA-MB-231. Throughout the paper, the studied sample is the crude extract obtained from *E. longifolia* leaf herbal infusion which is labelled as ELH.

II. MATERIAL AND METHOD

A. Plant collection and drying

The leaves of *Eurycoma longifolia* were collected from Kota Kinabalu in Sabah which located in East Malaysia. The leaves undergo steam blanching for 30 sec before ground and dried using freeze drying method.

B. Herbal infusion preparation

To simulate typical brewing conditions, an herbal infusion was created through a hot water extraction process. Precisely measured amounts of 2.0 g of dried unfermented and fermented leaves were steeped in hot water (98 ± 2□C) with continuous stirring at 300 rpm for 2 minutes using a magnetic stirrer. The mixture was then allowed to steep for an additional 10 minutes and subsequently filtered through a Whatman No.4 filter paper. The resulting filtered infusions were frozen at -20˚C and subjected to a 48-hour drying process at -50˚C and 0.125mbar under vacuum conditions using a freeze drier. Each 1.0 g of the lyophilized material, denoted as the crude extract, was mixed with 1.0 ml of dimethyl sulphoxide (DMSO) and stored at -20˚C for further testing.

C. Cell culture

The MDA-MB-231 human breast cancer cells were acquired from the American Type Culture Collection (ATCC, USA). Cultivation and maintenance were carried out in T75 flasks using RPMI 1640 growth medium supplemented with a combination of fetal bovine serum (FBS) and penicillin/streptomycin in a ratio of 100:10:1 (v/v/v). The cells were incubated at 37°C in a humidified environment with 5% carbon dioxide (CO2). Upon reaching confluence or near-confluence density, the cells were harvested using trypsin. Subsequently, cell counting was performed utilizing a 0.0025 mm2 hemocytometer and a 0.4% trypan blue exclusion assay.

D. Combinatorial MTT assay

The cell line was initially seeded at 1 x 10^3 cells per well and incubated for 24 hours at 37 °C in a 5% CO2 environment. After this period, the growth medium was replaced with ELH and tamoxifen as stated in Table 1. For the negative

control, cells were treated with only the growth medium.

TABLE 1
Combination of ELH and tamoxifen.

ID	Tamoxifen (ug/ml)	ELH (ug/ml)	ID	Tamoxifen (ug/ml)	ELH (ug/ml)
C-01	2.5	25	C-07	10.0	50
C-02	5.0	25	C-08	15.0	50
C-03	10.0	25	C-09	2.5	100
C-04	15.0	25	C-10	5.0	100
C-05	2.5	50	C-11	10.0	100
C-06	5.0	50	C-12	15.0	100

Following another 24h of incubation, MTT solution (0.5 mg/ml) was added for 3 hours. Then, DMSO (100 µl) was used to dissolve formazan crystals within viable cells. The plates were shaken for 30 minutes in the dark at room temperature (25 ± 2 °C), and absorbance was measured at 540 nm using an ELISA reader.

$$Percentage\ of\ cell\ viability\ (\%) = \frac{Optical\ density\ of\ sample}{Optical\ density\ of\ control} X\ 100\%$$

The combination index (CI) is a valuable metric used to assess the interaction between two drugs when administered together. Calculated as the ratio of the doses of each drug in combination to the doses that produce the same effect individually, the formula is given by:

$$CI = (a/A) + (b/B)$$

where "a" represents the small dose of the first drug combined with "b," the small dose of the second drug. Similarly, "A" denotes the large dose of the first drug that achieves the same effect, and "B" signifies the large dose of the second drug that produces an equivalent outcome.

III. RESULT AND DISCUSSION

Synergism is described as a positive interaction arising from the combination of two agents, leading to a collective inhibitory effect on the targeted organisms that exceeds the sum of their individual effects [19]. When the combination index (CI) equals 1, it indicates an additive effect, suggesting that the combined impact of the drugs is no greater or less than the sum of their individual effects. A CI less than 1 signifies synergy, where the combined effect is more potent than expected. Conversely, a CI greater than 1 indicates antagonism, revealing that the combined effect is less than anticipated.

Based on Fig. 1, the obvious decreasing pattern can be observed as the concentration of combination between the extract and tamoxifen increased. The cell viability percentage recorded high at 99.06% for the lowest concentration (2.5 µg/ml) of tamoxifen, whereas the cell viability percentage recorded lower at 66.75%, for the lowest concentration (25 µg/ml) of ELH. However, the lowest percentage of cell viability was shown by the combination of 2.5 µg/ml tamoxifen and 100 µg/ml ELH at 25.95%. A dose-dependent pattern is also evident with the increasing concentration of ELH in combination with the same tamoxifen concentration.

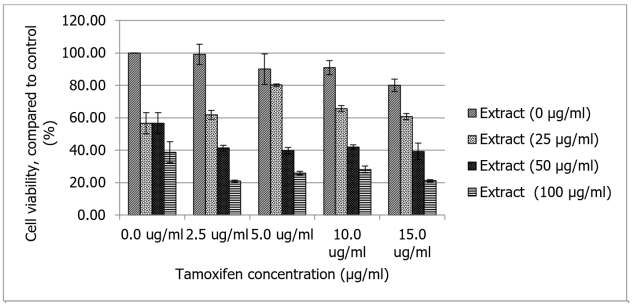

Fig. 1 Cell viability of various combination of ELH and tamoxifen.

Based on Table 2, most of the samples (C-05, C-06, C-07, C-08, C-09, C-10, C-11 and C-12) exhibit synergy, suggesting that the combination of tamoxifen and ELH at the given concentrations has a greater effect than expected based on the individual effects of each substance alone. Combinations of C-01, C-03, and C-04 show additive effects, while combination of C-02 shows antagonism. Only C-11 with the combination of 100 µg/ml ELH and 10 µg/ml tamoxifen showed strong synergy effect (CI = 0.66 ± 0.09) compared to the other combinations. These present findings suggested that synergistic effects can be obtained

for the combination of tamoxifen concentration ranged from 2.5 to 15.0 µg/ml and ELH concentration ranged from 50 to 100 µg/ml.

TABLE 2
Combination index of ELH and tamoxifen.

ID	Combination index (CI)	Effect
C-01	1.05 ± 0.24	Additive
C-02	2.14 ± 0.01	Antagonism
C-03	1.85 ± 0.08	Additive
C-04	1.65 ± 0.07	Additive
C-05	0.84 ± 0.11	Synergy
C-06	0.85 ± 0.24	Synergy
C-07	0.86 ± 0.14	Synergy
C-08	0.90 ± 0.07	Synergy
C-09	0.68 ± 0.11	Synergy
C-10	0.71 ± 0.14	Synergy
C-11	0.66 ± 0.09	Strong synergy
C-11	0.69 ± 0.01	Synergy

In similar study by Yaacob *et al.* [20], tamoxifen at the concentration of 15 µM induced cytotoxicity in dose-dependent manner within 24 hours and time-dependent manner from 12 hours to 48 hours for both MCF-7 and MDA-MB-231 cancer breast lines. Co-treatment with subfraction of *Strobilanthes crispus* (SCS) (8.5 µg/ml or 10.0 µg/ml) able to enhance the cell death. Notably, lower tamoxifen concentrations (2.5 and 5 µM) induced 90% cell death in MCF-7 cells when combined with SCS at 24 hours ($p < 0.001$). In MDA-MB-231 cells, the combination of 2.5 and 5 µM tamoxifen with SCS induced 85% and 95% cell death at 24 hours ($p < 0.001$).

In contrast, Jacobs and Browner's study [21] involving 20 women with early breast cancer revealed that the concurrent administration of Ginkgo biloba extract had no substantial impact on the pharmacokinetics of tamoxifen. The LC-MS/MS results indicated no significant differences in drug concentration and toxicity levels before and after a 3-week Ginkgo biloba extract treatment, administered at a dosage of 120 mg twice daily.

The presence or absence of synergistic effects between plant extracts and tamoxifen in cancer treatment arises from the distinctive chemical compositions of different plants and their interaction with tamoxifen's mechanisms of action. Synergy is more likely when plant compounds target similar or complementary molecular pathways relevant to cancer progression. Some plant extract and cancer medicine combination can exhibit effects beyond a singular target, influencing various functional or structural components within a cell, such as metabolites, receptors, enzymes, ion channels, transporters, nucleic acids, ribosomes, and proteins [22]. Factors such as antioxidant properties, anti-inflammatory effects, and influences on drug metabolism contribute to this complexity. Additionally, individual variability, tumor cell line specificity, and resistance mechanisms further contribute to varied outcomes.

IV. CONCLUSIONS

Based on the results, it is evident that specific combinations, particularly the mixture of 2.5 µg/ml tamoxifen and 100 µg/ml *E. longifolia* herbal infusion, exhibit the highest toxicity against breast cancer cells. The application of the combination index serves as a crucial quantitative metric for comprehending drug interactions. Over time, the pursuit of optimal combinations requires meticulous attention to factors such as concentration, dosage, and hormonal context to optimize the effectiveness of cancer therapy. Further research is warranted to elucidate the potential anticancer mechanisms of action, conduct bioavailability studies, and delve into toxicology aspects for a comprehensive understanding.

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REFERENCES

[1] Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne, M.,Vignat, J., Gralow, J. R., Cardoso, F., Siesling, S. & Soerjomataram, I. (2022). Current and future burden of breast cancer: global statistics for 2020 and 2040 *Breast*, Published online 2 September 2022; https://doi.org/10.1016/j.breast.2022.08.010

[2] World Health Organization. (2021). Malaysia. Globocan 2020.

[3] Debela DT, Muzazu SG, Heraro KD, Ndalama MT, Mesele BW, Haile DC, Kitui SK, Manyazewal T. New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Med. 2021 Aug 12;9:20503121211034366. doi: 10.1177/20503121211034366.

[4] Martinkovich, S., Shah, D., Planey, S.L., Arnott, J.A. (2014). Selective estrogen receptor modulators: tissue specificity and clinical utility. *Clinical Interventions in Aging,* 9:1437-52. doi: 10.2147/CIA.S66690. PMID: 25210448; PMCID: PMC4154886.

[5] Emons, G., Mustea, A., Tempfer, C. (2020). Tamoxifen and Endometrial Cancer: A Janus-Headed Drug. *Cancers (Basel),* 12(9):2535. doi: 10.3390/cancers12092535. PMID: 32906618; PMCID: PMC7564212.

[6] Ali M, Wani SUD, Salahuddin M, S N M, K M, Dey T, Zargar MI, Singh J. Recent advance of herbal medicines in cancer- a molecular approach. Heliyon. 2023 Feb 14;9(2):e13684. doi: 10.1016/j.heliyon.2023.e13684. PMID: 36865478; PMCID: PMC9971193.

[7] Ali M, Wani SUD, Salahuddin M, S N M, K M, Dey T, Zargar MI, Singh J. Recent advance of herbal medicines in cancer- a molecular approach. Heliyon. 2023 Feb 14;9(2):e13684. doi: 10.1016/j.heliyon.2023.e13684. PMID: 36865478; PMCID: PMC9971193.

[8] Wang TY, Li Q, Bi KS. Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. Asian J Pharm Sci. 2018 Jan;13(1):12-23. doi: 10.1016/j.ajps.2017.08.004. Epub 2017 Aug 15. PMID: 32104374; PMCID: PMC7032191.

[9] Yu, M., Gouvinhas, I., Rocha, J. *et al.* Phytochemical and antioxidant analysis of medicinal and food plants towards bioactive food and pharmaceutical resources. *Sci Rep* **11**, 10041 (2021). https://doi.org/10.1038/s41598-021-89437-4

[10] Dias MC, Pinto DCGA, Silva AMS. Plant Flavonoids: Chemical Characteristics and Biological Activity. Molecules. 2021 Sep 4;26(17):5377. doi: 10.3390/molecules26175377. PMID: 34500810; PMCID: PMC8434187.

[11] Ullah A, Munir S, Badshah SL, Khan N, Ghani L, Poulson BG, Emwas AH, Jaremko M. Important Flavonoids and Their Role as a Therapeutic Agent. Molecules. 2020 Nov 11;25(22):5243. doi: 10.3390/molecules25225243. PMID: 33187049; PMCID: PMC7697716.

[12] Garcia-Oliveira P, Otero P, Pereira AG, Chamorro F, Carpena M, Echave J, Fraga-Corral M, Simal-Gandara J, Prieto MA. Status and Challenges of Plant-Anticancer Compounds in Cancer Treatment. Pharmaceuticals (Basel). 2021 Feb 14;14(2):157. doi: 10.3390/ph14020157. PMID: 33673021; PMCID: PMC7918405.

[13] Lee EL and Barnes J Journal of Primary Health Care 2022; 14(4): 380–382. doi:10.1071/HC22143.

[14] Rehman SU, Choe K, Yoo HH. Review on a Traditional Herbal Medicine, Eurycoma longifolia Jack (Tongkat Ali): Its Traditional Uses, Chemistry, Evidence-Based Pharmacology and Toxicology. Molecules. 2016 Mar 10;21(3):331. doi: 10.3390/molecules21030331. PMID: 26978330; PMCID: PMC6274257.

[15] Bhat, R., Karim, A.A.. Tongkat Ali (*Eurycoma longifolia* Jack): A review on its ethnobotany and pharmacological importance, Fitoterapia, Volume 81, Issue 7, 2010, Pages 669-679, ISSN 0367-326X, https://doi.org/10.1016/j.fitote.2010.04.006.

[16] Al-Salahi, O. S. A., Zaki, A.H., Chan, K. L., Shah, A. M., Al-Hassan, F., Abdullah, W. Z. & Yusoff, N. M. 2013. In vitro anti-proliferative and apoptotic activities of Eurycoma longifolia Jack (Simaroubaceae) on HL-60 cell line. Tropical Journal of Pharmaceutical Research February, **12**(1):57-61.

[17] Tee, T. T. & Azimahtol, H. L. P. 2005. Induction of apoptosis by Eurycoma longifolia Jack extracts. Anticancer research, **25:**2205-2214.

[18] Mahfudh, N. & Pihie, A. H. L. 2006. Eurycomanone Exerts Antiproliferative Activity via Apoptosis in HeLa Cells. Bandung, Indonesia: International Conference on Mathematics and Natural Sciences (ICMNS).

[19] Levinson W & Jawetz E. (2002). Medical microbiology and immunology: Examination and board review. International. 7th ed., Lange Medical Books/McGraw-Hill, New York.

[20] Yaacob, N.S., Kamal, N.N.N.M. & Norazmi, M.N. Synergistic anticancer effects of a bioactive subfraction of *Strobilanthes crispus* and tamoxifen on MCF-7 and MDA-MB-231 human breast cancer cell lines. *BMC*

[21] Jacobs, B. P., and Browner, W. S. (2000). Ginkgo Biloba: A Living Fossil. Am. J. Med. 108 (4), 341–342. doi:10.1016/s0002-9343(00)00290-4Complement Altern Med 14, 252 (2014). https://doi.org/10.1186/1472-6882-14-252

[22] Pezzani, R.; Salehi, B.; Vitalini, S.; Iriti, M.; Zuñiga, F.A.; Sharifi-Rad, J.; Martorell, M.; Martins, N. Synergistic Effects of Plant Derivatives and Conventional Chemotherapeutic Agents: An Update on the Cancer Perspective. *Medicina* **2019**, *55*,110.https://doi.org/10.3390/medicina55040110.

