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Exploring the therapeutic potential of *Selaginella doederleinii* extract for cervical cancer treatment through network pharmacology and *in vitro* studies

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ABSTRACT
Cervical cancer is a significant health issue in Indonesia, with 36,633 cases reported in 2020. Chemotherapy, especially with doxorubicin, is a primary treatment but causes adverse effects such as cardiotoxicity, hair loss, nausea, vomiting, and immunosuppression. Combining chemotherapy with chemopreventive agents can enhance efficacy and reduce side effects. *Selaginella doederleinii*, known for its biflavonoid compounds, has potential chemopreventive activity. This study investigated the chemopreventive mechanism of *Selaginella doederleinii* ethanol extract (EESD) as a co-chemotherapy agent for HeLa cells through *in silico* and *in vitro* approaches. High-performance liquid chromatography analysis identified amentoflavone and 2,3-dihydro-3,3-diphenylapigenin as key compounds in EESD. Protein–protein interaction network analysis revealed 24 target receptors associated with cervical cancer, with ESR2 and HSP90AA1 being upregulated and involved in chemical carcinogenesis, estrogen signaling, cancer pathways, and endocrine resistance. EESD exhibited moderate cytotoxic activity (IC_{50} = 367.89 µg/ml) and demonstrated chemopreventive properties. The mechanism of EESD is presumed to involve the inhibition of key regulatory proteins in cervical cancer, namely ESR2 and HSP90AA1. Furthermore, EESD exhibited a slight synergistic effect with doxorubicin, with a combination index value of 0.85870. This study is the first to explore the chemopreventive potential of EESD along with its underlying mechanism, highlighting its role as a co-chemotherapy agent.

1. INTRODUCTION

Cervical cancer continues to be a major contributor to cancer-related deaths among women globally, with an estimated 604,000 new cases and 342,000 fatalities reported in 2020 [1]. Cervical cancer is a type of cancer caused by abnormal cell development, primarily triggered by the Human Papillomavirus (HPV) [2]. Standard therapeutic approaches commonly rely on chemotherapy, particularly doxorubicin; however, its effectiveness is often constrained by serious adverse effects, including fatigue, nausea, reduced appetite, and cardiotoxicity [3]. The cumulative toxicity and the emergence of drug resistance underscore the urgent need for new strategies to enhance both the efficacy and tolerability of treatment.

One promising approach to improving cancer treatment outcomes is the development of co-chemotherapy regimens that incorporate natural products as adjuvant agents. *Selaginella doederleinii* has emerged as a potential co-chemotherapeutic candidate due to its rich content of anticancer biflavonoids, including amentoflavone, robustaflavone, hinokiflavone, apigenin, sequoiaflavone, methoxyflavone, and thevetiaflavone [4]. A study by Ningrum *et al.* [5] demonstrated through *in silico* analysis that amentoflavone and 3,8-biapigenin exhibit strong binding affinity to STAT3, a key protein in cervical cancer progression, suggesting their potential as chemopreventive agents [5]. Additionally, several studies have reported that *S. doederleinii* possesses antioxidant and antiproliferative activities based on *in vitro* evaluations. The ethanol extract of *Selaginella doederleinii* (EESD) has demonstrated cytotoxic

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activity against various cancer cell lines, indicating its potential as a chemopreventive or adjunct chemotherapeutic agent [6]. However, the comprehensive mechanism by which EESD may enhance the efficacy of conventional chemotherapy agents such as doxorubicin remains largely unexplored.

The mechanism of anticancer action of EESD in preventing cervical cancer progression, particularly in combination with doxorubicin, remains poorly understood at the level of biological systems and molecular targets. To bridge this gap, a network-based approach was employed to investigate the interactions between bioactive compounds and disease-associated targets. Network pharmacology enables the mapping of complex relationships among drugs, targets, and diseases, allowing a more holistic understanding of how compounds exert their effects within biological systems [7]. In this study, an integrative strategy was employed, combining target prediction through bioinformatics with *in vitro* cytotoxicity assays to investigate the potential of EESD as a co-chemotherapeutic agent. To support this, expression profiling and survival analysis of key molecular targets were conducted using clinical databases, helping to identify those most relevant to cervical cancer outcomes [8]. Furthermore, the combinatory cytotoxic effect of EESD and doxorubicin (EESDox) was evaluated using the MTT assay on HeLa cells, providing functional insights into their potential synergistic activity.

2. MATERIALS AND METHODS

2.1. Extraction of *S. doederleinii*

The extraction method used was maceration, which involves immersing the plant powder in a solvent at room temperature [9]. To prepare the extract, 0.5 kg of *S. doederleinii* plant Simplicia powder was soaked in 5 l (1:10) of 70% ethanol solvent. Evaporation of the filtrate was carried out using a rotary evaporator at 100 rpm and 50°C, resulting in a viscous, concentrated extract free of solvent residues [10].

2.2. Identification test of compounds using high-performance liquid chromatography (HPLC) method

EESD (100 mg) was first dissolved in 10 ml of 70% ethanol and then vortexed. The mobile phase used to test the content of the bioflavonoid compounds consisted of Aquadest containing 0.5% acetic acid (v/v) (A) and acetonitrile (B) in a 60:40 ratio. The stationary phase utilized was a reversed-phase C18 column with dimensions of 250 × 4.6 mm and a particle size of 5 µm [11]. The flow rate was maintained at 500 µl/min, with an injection volume of 5 µl. Detection was carried out using a UV detector set at 270 nm, and chromatographic data were recorded over 60 minutes to determine the optimal retention time (Rt) peak.

2.3. Target fishing

To identify protein targets involved in the pathogenesis of cervical cancer, the keyword "cervical cancer" was used to search the GeneCards (https://www.genecards.org) and NCBI (https://www.ncbi.nlm.nih.gov/) databases. Disease-related protein targets and bioactive compounds were then imported into the PharmMapper database (http://www.lilab-ecust.cn/pharmmapper/) using a standard fit score threshold of >0.6. Canonical SMILES of the compounds were further analyzed using TargetNet (http://targetnet.scbdd.com/calcnet/calc_text/) with the species set to Homo sapiens and a probability cutoff of >0.6 [12]. Data from PharmMapper and TargetNet are combined to get potential protein targets. Potential protein targets were then identified by taking the intersections of the Venn diagrams between the compound target molecules and cervical cancer.

2.4. Construction of protein–protein interaction (PPI) network

The construction of the PPI network was performed by inputting the candidate genes into the STRING database (https://string-db.org/) using the highest confidence score threshold of 0.9 [13]. The results from the PPI network were analyzed and visualized using Cytoscape v 3.9.1 [14].

2.5. Enrichment analysis

Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontology (GO) function against common targets between diseases and drugs was analyzed using the DAVID Tool (https://david.ncifcrf.gov). Hits with p-value ≤0.05 are then used to create GO histograms and KEGG signal paths in bubble maps. KEGG bubble map with top values displayed with the help of an online bioinformatics tool (https://www.bioinformatics.com.cn/) [15].

2.6. Gene expression analysis

To compare gene expression levels between normal cervical tissue and cervical cancer, expression data were analyzed using the UALCAN platform (http://ualcan.path.uab.edu/analysis.html) [16]. The threshold for determining statistical significance is <0.05.

2.7. Cytotoxicity test using the 3-(4,5-dimetil-2tiazolil)-2,5-difenil-2H-tetrazolium bromida assay method

HeLa cells were cultured using a complete medium containing fetal bovine serum, Penicillin-streptomycin, fungizone, and Roswell Park Memorial Institute sterile liquid medium. Cells were cultured in small tissue flasks by incubation at 37°C [17]. Cells were seeded into each well of a 96-well plate at a density of 5×10^4 cells per 100 µl and incubated for 24 hours. Subsequently, 100 µl of culture medium containing various concentrations of EESDox was added to each well [18]. 3-(4,5-dimetil-2tiazolil)-2,5-difenil-2H-tetrazolium bromida (MTT) reagent was added, and formazan crystals were formed. The absorbance was then measured using an ELISA reader at a wavelength of 595 nm [19].

2.8. Combination cytotoxicity test with chemotherapeutic agents

Concentration series of EESDox were prepared at ½ IC_{50}, ¼ IC_{50}, 1/8 IC_{50}, and 1/16 IC_{50}. In the combination group, EESDox was added in separate steps with replications. Single-treatment groups received EESDox. Controls included cell control and media control. After a CO_2 incubation for 24–48 hours, MTT reagent was added, and formazan crystals were formed. Absorbance was read at 595 nm, and cell viability

and Combination Index (CI) were calculated using CompuSyn (ComboSyn, Inc, Paramus, NJ) [19].

3. RESULTS

3.1. Extraction

Extracting 500 g of finely powdered *S. doederleinii* plant material involved utilizing the maceration method with 5 l of 70% ethanol solvent (1:10 ratio). This process yielded a concentrated extract weighing 27.3 g, exhibiting a color resembling dark brown with a hint of greenish black. The obtained yield from the extraction was 5.46%.

3.2. Identification test of compounds using HPLC method

The results of the qualitative test of ESSD using the HPLC method were analyzed based on the Rt value. The results of the Rt value obtained from EESD were compared with the standard Rt value from previous studies. Based on Figure 1 and Table 1, the Rt value obtained from EESD is found in peaks 6 and 7 with Rt values of 11,907 and 16,202. Based on the analysis results, EESD in this study is suspected to contain 2 types of biflavonoid groups, namely amentoflavone and 2,3-Dihydro-3,3-biapigenin compounds. These results were obtained by comparing the Rt value of EESD with the Rt value of standard compounds from research (Fig. 2) [11] amentoflavone with Rt value 11–12 and 2,3-Dihydro-3,3-biapigenin with Rt value 16–17.

3.3. Target fishing

Target fishing was conducted by integrating data from multiple databases (TargetNet, PharmMapper, GeneCards, and NCBI) to identify potential protein targets of the compounds and their association with cervical cancer. By combining genes from these sources and removing duplicates, we identified 3,713 cervical cancer-related proteins, 74 proteins interacting with amentoflavone, and 54 proteins related to 2,3-dihydro-3,3-biapigenin. Through Venn diagram analysis, 24 overlapping proteins were found to be associated with both compounds and cervical cancer.

3.4. Identifying amentoflavone and 2.3-Dihidro-3.3-biapigenin targets, and intersection with cervical cancer

The PPI network of 24 cervical cancer-related targets, including amentoflavone and 2,3-dihydro-3,3-biapigenin, was constructed using STRING analysis (Fig. 3). The resulting network consisted of 24 nodes and 83 edges. Degree centrality (DC) analysis was initially performed to filter out target nodes with degrees greater than the average degree (6.92), yielding 13 nodes and 52 edges. Subsequently, a second filtering step was conducted using three topological parameters DC, betweenness centrality (BC), and closeness centrality (CC) to identify key targets with critical roles in cervical cancer. Based on the average values of DC (8), BC (4), and CC (0.7626), a refined subnetwork of 5 nodes and 10 edges was obtained (Fig. 4) and used for subsequent KEGG pathway enrichment analysis.

3.5. GO and KEGG enrichment analysis

GO enrichment analysis was performed on the five primary target proteins, covering three categories: biological process (BP), molecular function (MF), and cellular component (CC). The results were filtered based on statistical significance (p-value <0.05), yielding 20 BP terms, 17 MF terms, 7 CC terms, and 9 KEGG pathways. For presentation purposes, only the top

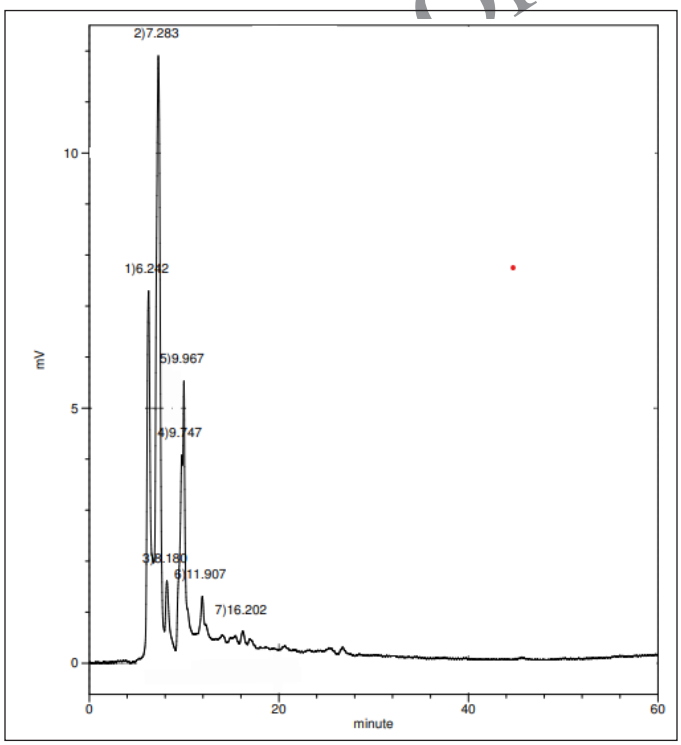

Figure 1. EESD chromatogram.

Table 1. Rt comparison of EESD to the previous research.

Compounds	Rt of previous research [8] (Minutes)	Rt of EESD (Minutes)
Amentoflavone	11–12	11.907
Robustaflavone	13	-
2.3-Dihidro-3.3-biapigenin	16–17	16.202
3,3-Binaringenin	18–19	
Delicaflavone	22–23	
Heveaflavone	41–42	
7,4′,7″,4″ ′-tetra-O-methyl-amentoflavone	47	

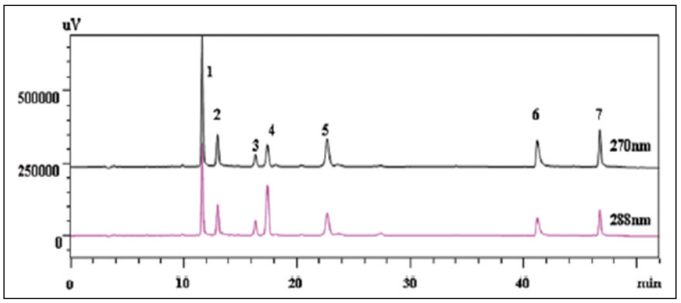

Figure 2. Chromatogram of marker compound according to Li *et al.* [11].

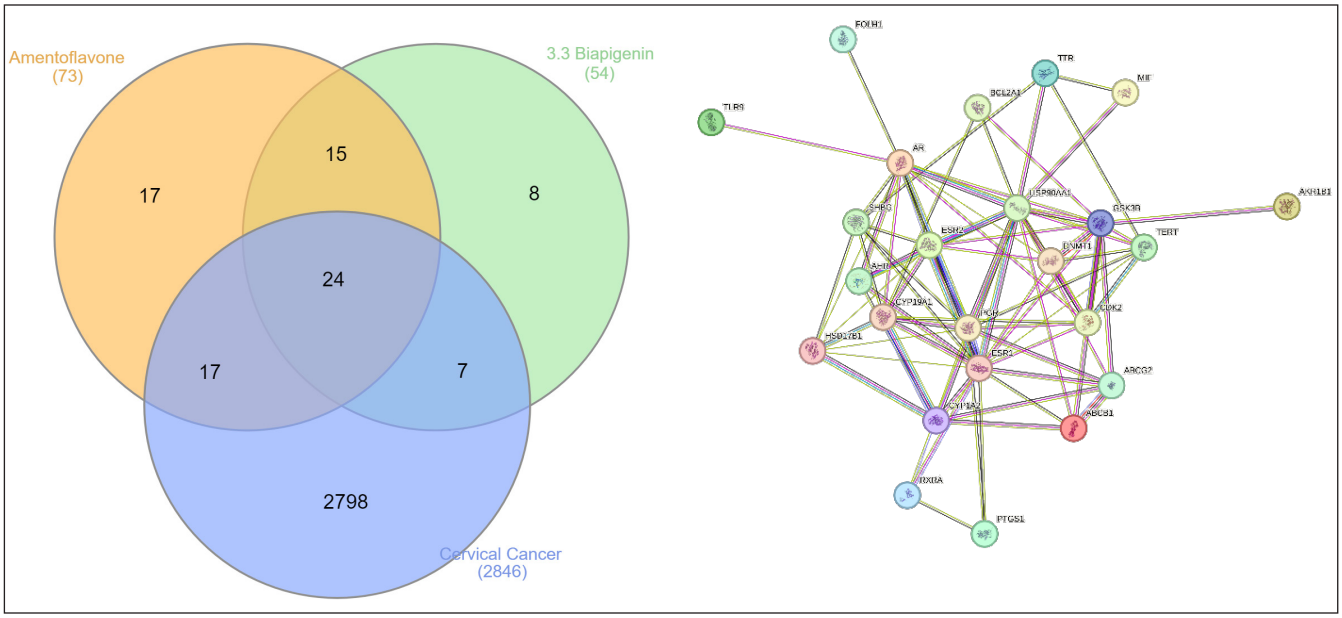

Figure 3. PPI network using STRING.

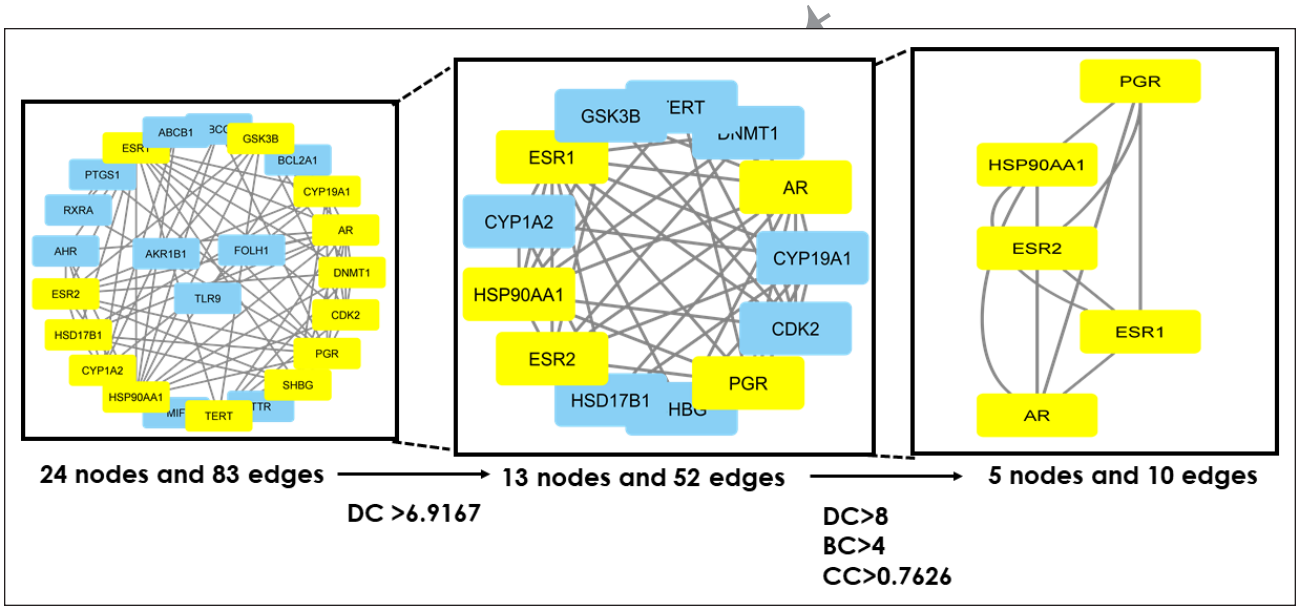

Figure 4. The inter-protein interaction analysis was conducted using the CytoNCA plug-in in two screening steps. In the final results, five core targets—PGR, HSP90AA1, ESR2, ESR1, and AR—were identified based on three centrality parameters: DC, BC, and closeness centrality (CC).

seven terms from BP, MF, and KEGG analyses were selected. In the CC category, only the top four terms were included, as only four met the significance threshold. Detailed results are presented in Tables 2 and 3. The mechanistic relationship between the five core target proteins and cervical cancer is illustrated in Figure 6.

3.6. Prognostic value of the potential target genes of amentoflavone and 2.3-Dihidro-3.3-biapigenin

Analysis of gene expression levels and critical survival rates was performed in cervical cancer using the TCGA dataset with UALCAN tools. The purpose of analyzing gene expression levels is to obtain biomarker genes that appear very prominent during the course of the disease. As shown in Figure 7, only ESR2 and HSP90AA1 exhibited elevated expression levels in cervical cancer patients compared to normal controls. In contrast, AR, PGR, and ESR1 showed decreased expression in cervical cancer patients.

Therefore, genes with higher expression in cervical cancer were further analyzed in relation to the survival rate of cervical cancer patients. For 6,000 days, the overall survival of cervical cancer patients was analyzed using Kaplan-Meier survival. Figure 8 demonstrates that the expression levels of ESR2 and HSP90AA1 are negatively correlated with overall

Table 2. Top 7 KEGG pathway classification of targets.

KEGG pathways	Count	%	*p*-value	Genes
Chemical carcinogenesis receptor activation	5	100	3.4045E-07	AR, HSP90AA1, PGR, ESR1, ESR2
Estrogen signaling pathway	4	80	1.5E-05	HSP90AA1, PGR, ESR1, ESR2
Pathways in cancer	4	80	8.33E-04	AR, HSP90AA1, ESR1, ESR2
Breast cancer	3	60	0.001634	PGR, ESR1, ESR2
Prolactin signaling pathway	2	40	0.031747	ESR1, ESR2
Prostate cancer	2	40	0.043619	AR, HSP90AA1
Endocrine resistance	2	40	0.0440568	ESR1, ESR2

Table 3. Top seven GO.

KEGG pathways	Count	%	*p*-value	Genes
Biological processes				
Nuclear receptor-mediated steroid hormone signaling pathway	3	60	3.4045E-07	AR, HSP90AA1, PGR, ESR1, ESR2
Cellular response to estrogen stimulus	3	60	1.5E-05	HSP90AA1, PGR, ESR1, ESR2
ER signaling pathway	3	60	8.33E-04	AR, HSP90AA1, ESR1, ESR2
Tertiary branching involved in mammary gland duct morphogenesis	2	40	0.001634	PGR, ESR1, ESR2
Cell-cell signaling	3	60	0.031747	ESR1, ESR2
Signal transduction	4	80	0.043619	AR, HSP90AA1
Positive regulation of transcription by RNA polymerase II	4	80	0.0440568	ESR1, ESR2
MF				
Estrogen response element binding	4	80	7.338E-10	AR, PGR, ESR1, ESR2
DNA binding	4	80	8.21E-04	AR, PGR, ESR1, ESR2
Nuclear receptor activity	4	80	8.2603E-08	AR, PGR, ESR1, ESR2
Nuclear steroid receptor activity	3	60	8.135E-06	PGR, ESR1, ESR2
Enzyme binding	4	80	2.80E-05	AR, PGR, ESR1, ESR2
Transcription coactivator binding	3	60	3.62E-05	AR, PGR, ESR1
Nuclear ER activity	2	40	8.28 E-04	ESR1, ESR2
Cellular component				
Chromatin	4	80	6.48E-04	AR, PGR, ESR1, ESR2
Nucleoplasm	5	100	0.00138	AR, PGR, ESR1, ESR2
Protein-containing complex	3	60	0.006149	AR, HSP90AA1, ESR1
Nucleus	5	100	0.00763	AR, HSP90AA1, PGR, ESR1, ESR2

survival in cervical cancer patients. Specifically, patients with high expression levels of these genes (green curves) exhibit significantly poorer survival outcomes compared to those with low expression levels (orange curves). For instance, patients with elevated HSP90AA1 expression show a survival duration of less than 6,000 days with a survival probability below 0.5. Similarly, although patients with high ESR2 expression may survive beyond 6,000 days, their survival probability also remains below 0.5. Furthermore, to analyze more deeply the role of *S. doederleinii* in cervical cancer, further analysis was carried out by conducting an *in vitro* anticancer activity test on HeLa cells, and analyzing the effect of the combination of chemotherapy with doxorubicin based on the CI.

3.7. Cytotoxicity test using the MTT assay method

The data shown in Table 4 shows that EESD has moderate cytotoxic properties with an IC_{50} value of 367.89 µg/ml, because the IC_{50} value is in the range of 100–1,000 µg/ml. Based on the IC_{50} results, it shows that EESD requires a concentration of 367.89 µg/ml to inhibit 50% of HeLa cervical cancer cells. The test results of doxorubicin chemotherapy agents have an IC_{50} value of 2.45 µg/ml, which is included in the potential or very strong cytotoxic category. In addition to analyzing the IC_{50} value to determine the cytotoxic effect of a compound, morphological observations of HeLa cells were also observed using an inverted microscope with 40× magnification in Figure 9.

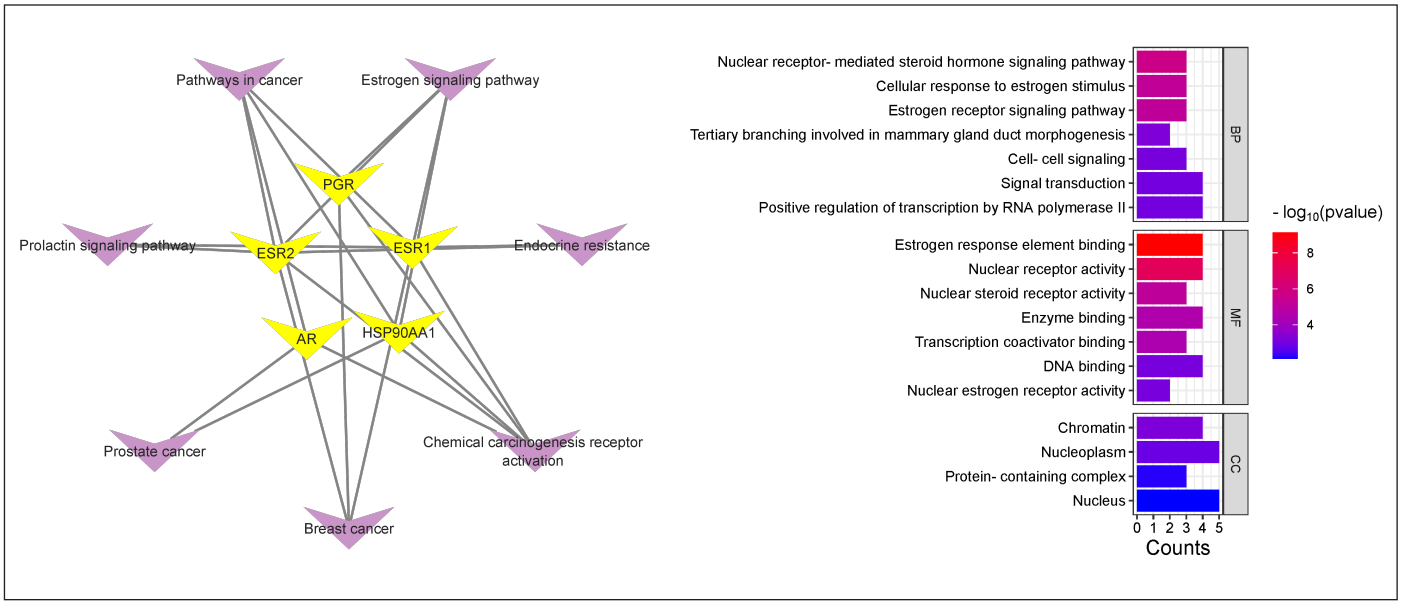

Figure 5. KEGG pathway analysis of target genes shortlist by top seven representative pathways according to gene (a), GO search results of potential targets of amentoflavone and 2.3-Dihidro-3.3-biapigenin on cervical cancer, including BP, cellular components and MFs (b).

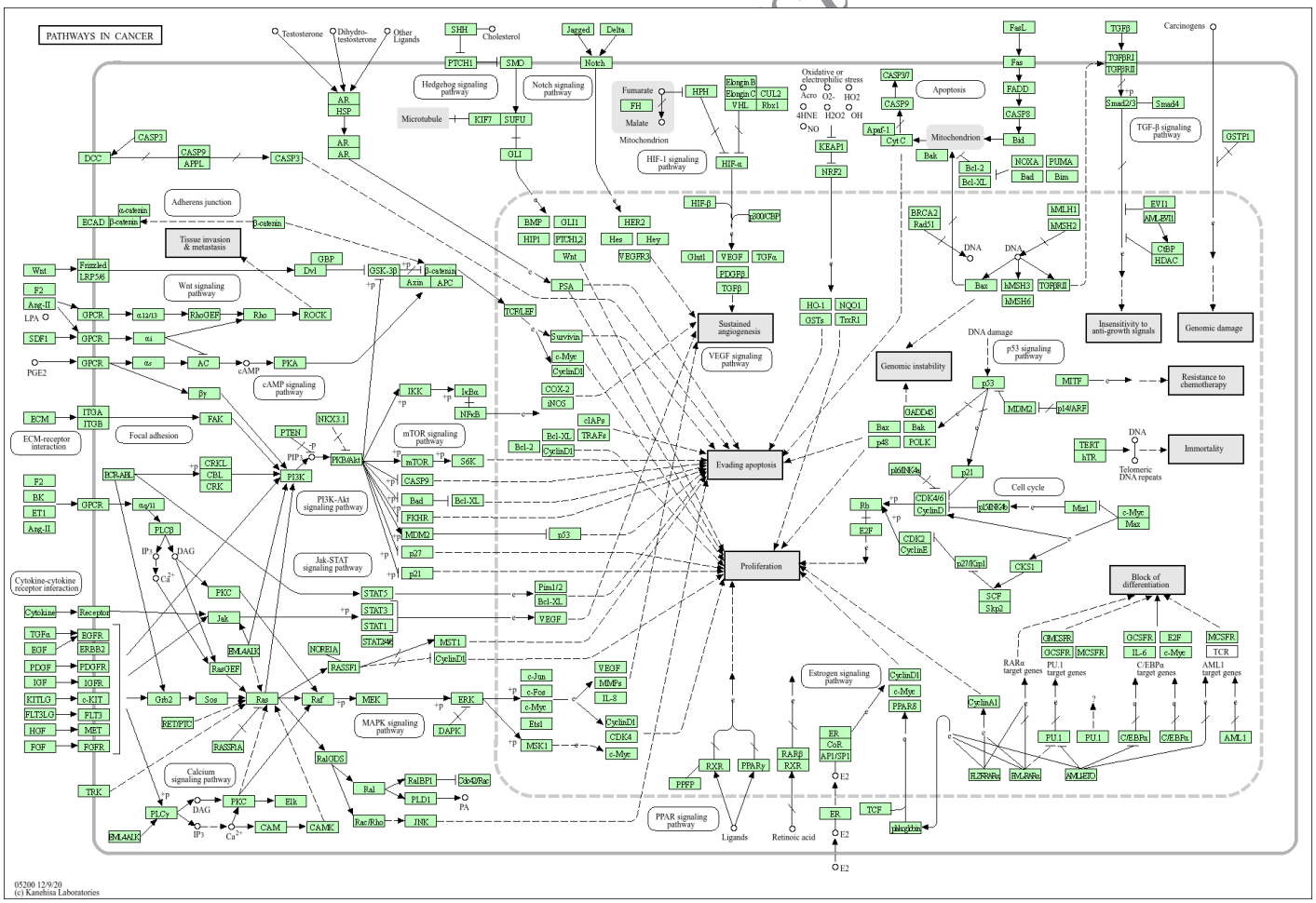

Figure 6. Major protein target signaling pathway in cancer [15,20].

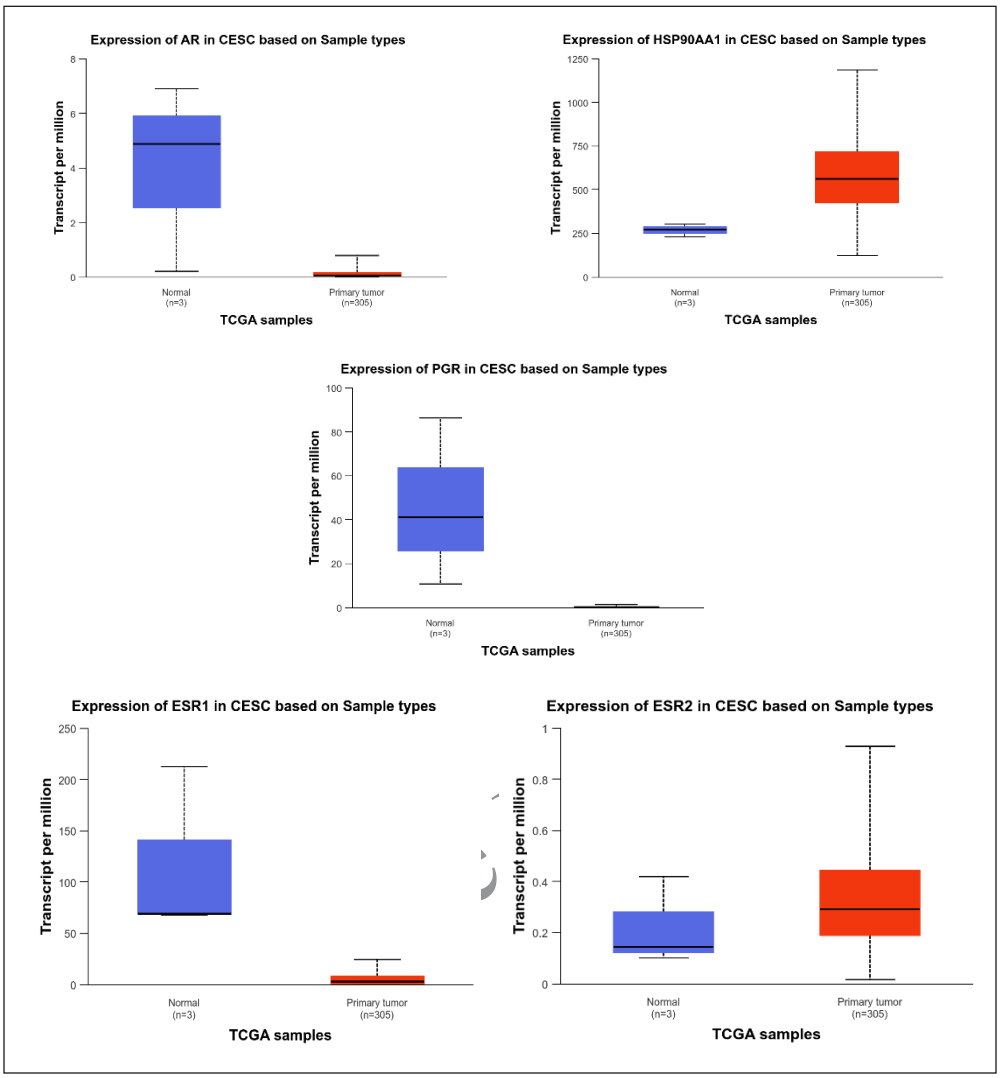

Figure 7. The expression levels of key genes in normal (blue) and cervical cancer (CESC) (orange) samples are illustrated.

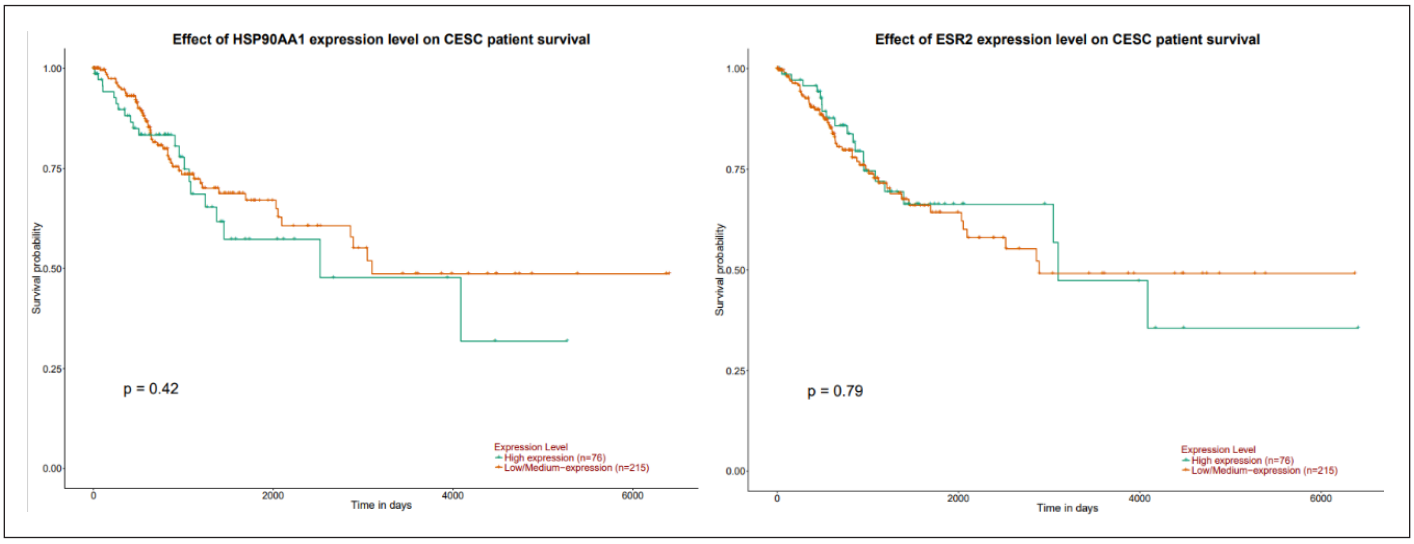

Figure 8. Kaplan–Meier survival analysis showing the correlation between gene expression levels and overall survival in CESC patients. Upregulated genes are depicted with green survival curves, while downregulated genes are shown in orange.

3.8. Combination cytotoxicity test with chemotherapeutic agents

MTT assay test data in the form of IC_{50} values of EESD test samples have moderate potential, so further development research is carried out to maximize the potential of EESD, namely by combining EESD with chemotherapeutic agents to increase its potential. Combination chemotherapy is the combination of compounds that are chemopreventive with chemotherapy agents. The parameter used in the co-chemotherapy test is the CI value. Based on Figure 10 and Table 5, the best combination value is obtained from the combination of 1/16 IC_{50} EESD (22.99 (μM) with 1/16 IC_{50} Doxorubicin (0.152 (μM) which has a value of 0.85870 which showing a strong synergistic effect and slight synergism. Based on Figure 11, EESD exhibited a gradual increase in cytotoxic effect with increasing doses; however, it did not reach the maximum effect (Fa = 1), even at higher concentrations, indicating relatively low cytotoxic potential as a single agent. In contrast, Doxorubicin demonstrated a high potency, showing substantial effects at very low doses, as reflected by Fa values approaching 1. Meanwhile, the combination of EESDox produced inconsistent effects, with some data points showing high Fa values while others remained low. This variability suggests that the combination has not consistently resulted in synergistic effects. This observation is consistent with the CI values ranging from 0.8 to 12, indicating a weak synergistic to strong antagonistic interaction. These findings suggest that although the combination of EESDox has not yet demonstrated

Table 4. Cytotoxic activity.

IC_{50} value (l)		Linear equation
EESD	367.89	$y = -0.1797x + 116.11$ $R^2 = 0.9589$
Doxorubicin	2.45	$y = -12.536x + 80.743$ $R^2 = 0.7905$

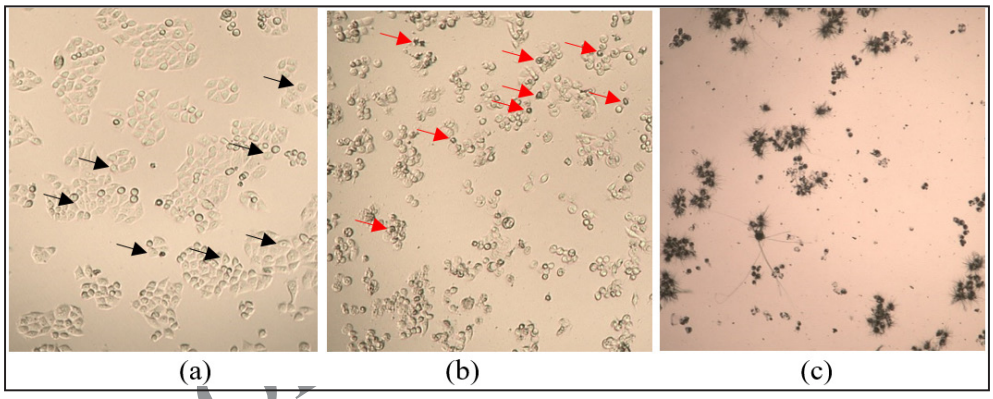

Figure 9. Morphological changes of HeLa cells on treatment with EESD (a) Before treatment, (b) After treatment, (c) After MTT reagent addition. (⟶) live cells; (⟶) dead cells.

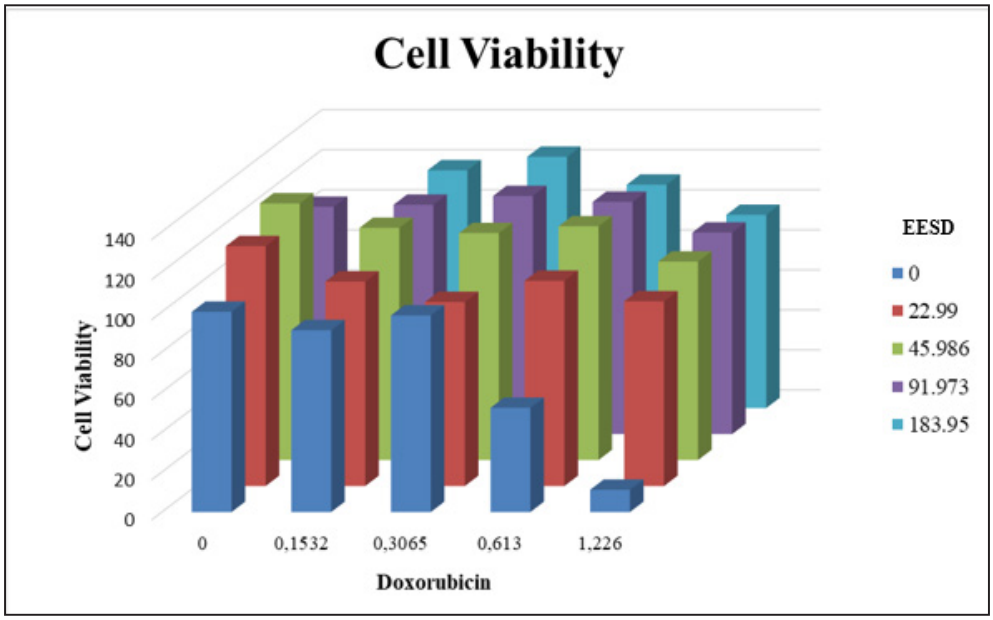

Figure 10. Cell viability chart.

Table 5. CI value of EESDox calculated using CompuSyn (ComboSyn, Inc, Paramus, NJ).

Concentration		Doxorubicin (μM)			
		0.1532	0.3065	0.613	1.226
	22.99	0.85870	1.23517	1.13916	5.01021
EESD(μM)	45.986	1.42725	1.55952	1.71825	12.6226
	91.973	3.85718	2.79150	2.78284	3.05421
	183.95	5.27024	5.06420	5.93542	7.10180

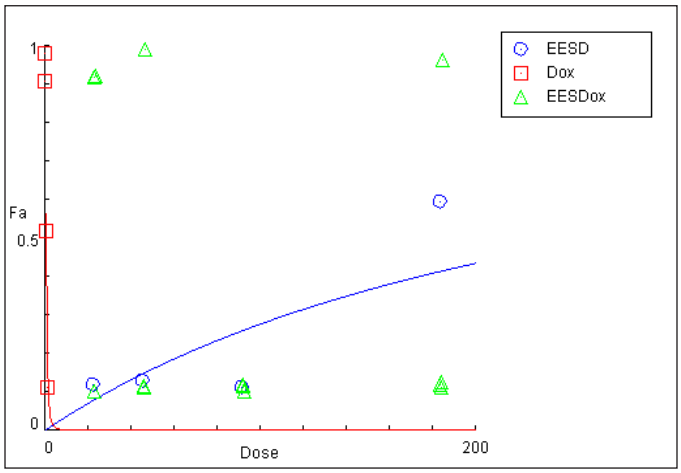

Figure 11. Dose-effect curves of EESD, doxorubicin, and the EESDox combination.

optimal effectiveness, the mild synergistic effects observed at lower concentrations warrant further investigation, particularly on normal cells, to assess safety and to optimize the combination dosing strategy.

4. DISCUSSION

Cervical cancer is a disease primarily caused by HPV [21]. On HPV DNA, the E6 and E7 oncoproteins disrupt the host cell cycle. Specifically, E6 interferes with the tumor suppressor protein p53, while E7 disrupts the retinoblastoma protein. Additionally, E5 proteins may contribute to immune evasion [22]. Although many cancer treatments are available, they often have significant side effects [23]. Chemotherapy, a commonly employed treatment for various types of cancer, can also harm healthy cells, leading to adverse effects such as nausea, hair loss, vomiting, fatigue, and, in severe cases, fatal outcomes. Chemotherapy relies on drugs that selectively target tumor cells, primarily through genotoxic mechanisms involving reactive oxygen species [24]. However, this process also damages normal cells [25]. In traditional Chinese medicine, *S. doederleinii* is used as an herb to treat diseases such as chorionic carcinoma, nasopharyngeal carcinoma, and various types of cancer [26]. Extracts from *S. doederleinii* have potential as raw materials for anticancer drug development due to their biflavonoid content [27]. Biflavonoids are known for their antioxidant, cancer chemopreventive, anti-inflammatory, and antimicrobial activities [28].

Maceration is a process where the solvent penetrates the cell wall and dissolves active substances due to concentration gradients. This causes the concentrated solution inside the cell to diffuse outward until a balance is reached between the solution inside and outside the cell [29]. In this study, the yield obtained from maceration was 5.46%, which is relatively low. This could be due to the incomplete extraction of active substances that are insoluble in ethanol. To ensure the quality of the extract, the biflavonoid content in EESD was analyzed using HPLC, a widely used method. HPLC method has been widely used in previous studies. Research conducted by Yao *et al.* [30] on the analysis of total biflavonoids from *S. doederleinii* extract using HPLC with an additional quadrupole time of flight mass spectrometry system, which showed the results of 20 types of biflavonoids analyzed in the *S. doederleinii* extract. In addition, similar research conducted by Li *et al.* [11] using the HPLC method related to the analysis of 7 types of biflavonoids contained in *S. doederleinii*. In this study, it refers to the research conducted by Li *et al.* [11] using the same mobile phase and stationary phase to identify biflavonoid compounds, namely phase A [Aquadest (0.5% acetic acid v/v)] and B (acetonitrile) with a ratio of (55:45).

Observations were made by comparing the Rt of the test sample and the Rt of the standard compound in the study [11]. The results indicate that EESD contains amentoflavone compounds with an Rt value of 11,907 and 2,3-Dihydro-3,3-biapigenin with an Rt value of 16,202. These results are close to the Rt values in previous research conducted by Li *et al.* [11], namely amentoflavone Rt 11-12 and 2,3-Dihydro-3,3-biapigenin Rt 16-17. Amentoflavone is the main compound contained in *S. doederleinii,* which has the activity to effectively inhibit various kinds of tumor cell proliferation but can also induce tumor cell apoptosis and differentiation [28]. Nonetheless, the receptors underlying the action of amentoflavone and 2,3-Dihydro-3,3-biapigenin on cervical anticancer mechanisms remain unclear. Therefore, this study evaluated several potential targets related to the mechanism of EESD content compounds, particularly amentoflavone and 2,3-Dihydro-3,3-biapigenin in preventing cervical cancer severity and its correlation with upregulated genes and survival rate, as well as their activity *in vitro* on HeLa cells and complementary treatment opportunities when combined with chemotherapeutic agents.

By cross-referencing cervical cancer-related targets obtained from TargetNet, PharmMapper, and GeneCards, a total of 24 potential targets for amentoflavone and 2,3-dihydro-3,3-biapigenin in cervical cancer therapy were identified. Based on the results of PPI network construction, as well as GO and KEGG enrichment analyses, five key receptor targets were selected due to their critical roles in cervical cancer: PGR, HSP90AA1, ESR2, ESR1, and AR. The involvement

of these proteins in relevant disease pathways is illustrated in Figure 5. Overall, all identified targets participate in cancer-related signaling pathways. Notably, five of the seven enriched pathways, chemical carcinogenesis: receptor activation, estrogen signaling pathway, pathways in cancer, breast cancer, prostate cancer, and endocrine resistance, were associated with the selected targets. All five receptors are implicated in the chemical carcinogenesis: receptor activation pathway, while HSP90AA1, ESR2, ESR1, and AR play roles in the broader "Pathways in Cancer" category. The chemical carcinogenesis: receptor activation pathway differentiates between genotoxic and nongenotoxic carcinogens. Genotoxic carcinogens exert their effects by directly damaging DNA, initiating carcinogenesis through DNA strand breaks, adduct formation, or other genetic alterations. In contrast, nongenotoxic carcinogens do not directly interact with DNA but instead promote tumor development through receptor-mediated or epigenetic mechanisms. One key nongenotoxic mechanism involves the activation of cellular receptors, which can lead to altered gene expression and cellular behavior contributing to carcinogenesis [27]. The estrogen signaling pathway plays a role in the development of female cancers, such as breast, ovarian, cervical, and other gynecological cancers [28]. Estrogen Receptor (ER) expression and uncontrolled ER signaling can lead to uncontrolled cell proliferation and cancer development [31].

Cervical squamous cell carcinoma (CESC) patients from the TCGA dataset were used because cervical cancer is often a squamous cell carcinoma arising from high-risk serotype-16 and 18 HPV infection [32]. Prognostic analysis of key genes was conducted using the UALCAN platform to evaluate the expression levels of target genes and their correlation with survival outcomes in CESC patients. This study reveals that ESR2 and HSP90AA1 may be significant targets of amentoflavone and 2,3-Dihydro-3,3-biapigenin in preventing cancer progression and complications, especially in CESC, as ESR2 and HSP90AA1 are upregulated in CESC and have important roles in patient survival. This study will reveal the mechanism of action of EESD when used as cervical cancer therapy, so to confirm the anticancer activity of EESD, a cytotoxic test was conducted on HeLa cervical cancer cells [33].

The MTT assay test is used to determine the potential of a drug as an anticancer through a cytotoxic mechanism. The parameter used in this study is the IC_{50} value, which describes the concentration of a sample in producing 50% cell proliferation inhibition. The smaller the IC_{50} value, the more potent the toxicity of a compound. The IC_{50} value of EESD against HeLa cells was 367.89 µg/ml, indicating that EESD required this concentration to inhibit 50% of HeLa cervical cancer cells. These results indicate moderate cytotoxic activity, because the IC_{50} value is between the range of 100–1,000 µg/ml, which can be used as a chemopreventive agent that is useful in preventing or inhibiting the division or development of cancer cells [34]. Figure 9 shows changes in HeLa cell morphology between each treatment. Living cells are round and there is a cell nucleus in the middle of the cell, which is small black, while dead cells are round and full black color. Figure 9 shows changes in HeLa cell morphology between each treatment. Living cells are round and there is a cell nucleus in the middle of the cell, which is small black, while dead cells are round and full black color. Cells before treatment are round and slightly oval, and the cell nucleus is still visible. After the treatment, the cell morphology changes into irregular shapes, small rounds, like shrinking and darkening, until the cell nucleus is no longer visible. The picture of changes in cell morphology illustrates that cell death has occurred due to treatment with the sample. Cell death is thought to occur in apoptosis with the characteristics, namely, there is pycnosis due to chromatin condensation. Cells will change size to become smaller due to cytoplasmic condensation, due to loss of intracellular fluid or loss of dehydration in cells [35].

Chemopreventive agents function as blocking agents during the initiation phase, preventing carcinogens from reaching their targets either by inhibiting interactions with macromolecular targets such as DNA, RNA, or proteins. Chemopreventive compounds can also act as suppressing agents, inhibiting malignant formation during the promotion or progression stages of a cell that was initiated earlier in the initiation phase [36]. The results of this *in vitro* test correlate with previous data, suggesting that EESD may function as a chemopreventive agent through its mechanism of action as a blocking agent, inhibiting ESR2 and HSP90AA1 proteins in the chemical carcinogen activation receptor pathway. Under pathological conditions, ESR2 plays a role in tumor development [37] and is considered a tumor suppressor gene, ESR2 is a tumor suppressor gene [36], ESR2 can also mediate the development of prostate cancer [38], colon cancer [39], ovarian cancer, and lung cancer [40], so ESR2 not only plays a role in cervical cancer but can also be used as a potential cancer target in various types of cancer [40–42]. As shown in Figure 6, the ESR2 protein in cancer pathways is involved in the estrogen signaling pathway at the proliferation stage, alongside cyclin D1. In cervical cancer, HSP90AA1 is upregulated, particularly in relation to chemotherapy and radiotherapy resistance [43]. Thus, HSP90AA1 may serve as a potential target for cervical cancer, especially in cases exhibiting resistance to chemotherapy and radiotherapy, with an inhibitory mechanism.

The selectivity of chemopreventive agents lies in their ability to specifically target cancer cells while sparing normal cells. This differs significantly from the mechanism of chemotherapy drugs, which attack both cancer and normal cells, leading to dangerous side effects [44]. Another strategy to enhance the effectiveness of chemopreventive agents and reduce the side effects of chemotherapy is combination therapy. In this study, a combination chemotherapy test was conducted using doxorubicin, which has cardiotoxic effects and poses a risk of death when used in high doses [45]. When chemotherapy drugs are combined with chemopreventive agents, they ideally produce a synergistic effect against cancer cells while maintaining tolerable toxicity, making the combination clinically more efficient than using chemotherapy agents alone [19].

Based on the IC_{50} value, doxorubicin in this study demonstrated strong cytotoxic potential, with an IC_{50} value of 2.45 µg/ml. The combination dose used was below the IC_{50} value, as it aimed to achieve a high anticancer effect at a lower dose, which could help reduce the unwanted side effects associated with the combination. CI analysis was conducted to assess

the effect of the combination, such as synergistic, additive, or antagonistic effects. The best combination was achieved with 1/16 IC_{50} of EESD and 1/16 IC_{50} of doxorubicin, resulting in a CI value of 0.85870, indicating a slight synergism effect. The combination of EESDox in this study produced CI values ranging from 0.8 to 12, indicating a spectrum of interactions from slight synergism to strong antagonism. The overall results suggest that the combination has not yet achieved optimal effectiveness. Based on the dose-response data (Fig. 10), the cell viability across all combination treatments ranged from 80% to 120%, indicating that the cytotoxic impact was generally low and in some cases even promoted cell proliferation. However, the slight synergism observed at low concentrations suggests a cytostatic effect, in which cell growth is inhibited without necessarily inducing cell death. This is further supported by the notion that the synergy score does not always correlate with a decrease in cell viability—synergistic interactions can still occur even when a high percentage of viable cells remains [46,47]. These findings imply that while the current combination may not be cytotoxic, it holds potential for further investigation, particularly in normal cells, to assess safety and explore optimized dosing strategies that could enhance the therapeutic effect or reduce the required dose of doxorubicin [48].

5. CONCLUSION

In conclusion, this study identified EESD as a potential source of biflavonoid compounds, specifically amentoflavone and 2.3-dihydro-3,3-biapigenin, which demonstrated chemopreventive properties. EESD exhibited moderate cytotoxicity against HeLa cervical cancer cells, with an IC_{50} value of 367.89 µg/ml. The mechanism of action of EESD is presumed to involve the inhibition of highly regulated proteins in cervical cancer, namely ESR2 and HSP90AA1, which are key players in the receptor activation pathway of chemical carcinogenesis, cancer pathways, and endocrine resistance. In addition, EESD exhibited slight synergism activity when combined with doxorubicin. Further *in vivo* and clinical studies are recommended to validate these findings and explore the broader therapeutic potential of EESD. Moreover, additional investigations using Western blot, qPCR, or siRNA knockdown assays may be conducted to validate the mechanism of EESD as a potential agent for cervical cancer treatment and include cytotoxicity assays in noncancerous cells to demonstrate selectivity and safety from EESD to reduce side effects.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, Indonesia (number 065/EC-EXEM-KEPK FKIK UMY/VII/2022).

11. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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