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Comparative metabolite profiling of *Eleutherine bulbosa* ethanol and water extracts by UPLC-MS/MS and their cytotoxic effects on T47D cells

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ABSTRACT

Eleutherine bulbosa is a plant renowned in the scientific literature for its multiple pharmacological properties. These effects are attributed to its phytochemicals, which include compounds from the naphthalene, anthraquinone, and naphthoquinone groups. The effectiveness and concentration of active biological components are greatly affected by the type of solvent selected for the extraction procedure. This study aims to compare the metabolite profiles of ethanol and water extracts from Dayak onion (*E. bulbosa*) and to assess their cytotoxic activities against cancer cells. Metabolite profiling was performed using an ultra-performance liquid chromatography-mass spectrometry/mass spectrometry instrument. The molecular formulas of the constituents were analyzed using MassLynx 4.1 software, while compound names and structures were identified through the PubChem database. The metabolite profiling revealed that the ethanol extract of *E. bulbosa* contained 32 compounds, while the water extract had 27. Analysis using a Venn diagram identified three common compounds in both extracts: 1,4-Naphthoquinone ($C_{10}H_6O_2$), L-lysine sulfate ($C_6H_{16}N_2O_6S$), and Epirizole ($C_{11}H_{14}N_4O_2$). Cytotoxicity assays indicated that the ethanol extract of *E. bulbosa* exhibits low anticancer potential with an IC50 value of 202.37 µg/ml. In contrast, the water extract does not exhibit anticancer activity with an IC50 of 1,020 µg/ml. However, the ethanol extract has shown efficacy in initiating programmed cell death and interfering with the cell division process in T47D cancer cells. These findings suggest that an ethanol solvent is more adept at extracting the bioactive components of *E. bulbosa*, underscoring its potential in anticancer therapy development. Nonetheless, further research is essential to elucidate the activity of these phytochemicals and to evaluate their toxicity toward normal cells.

INTRODUCTION

Natural substances offer a promising alternative for medical therapies in managing various health conditions. The bulb of the Dayak onion, *Eleutherine bulbosa* (*E. bulbosa*), a notable member of the Iridaceae family found in Southeast Asia, boasts a range of therapeutic benefits. Historically used by the Dayak people to remedy conditions including diabetes, breast cancer, blocked nasal passages, and reproductive health problems [1]. The bulb contains an abundance of chemical components such as phenolic compounds, flavonoids, naphthalene, anthraquinones, and naphthoquinones, all of which enhance its therapeutic capabilities [2]. Synonyms for *E. bulbosa include Eleutherine americana, Eleutherine palmifolia, and Eleutherine platifolia* [3]. Its documented uses span anticancer [4], antidiabetic [5], antibacterial [6], antifungal

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[7], antiviral [8], anti-inflammatory [9], dermatological [10], antioxidative [11], and fertility regulatory activities [3]. Despite extensive reports on *E. bulbosa's* active components, comparative analysis of these compounds in different solvent extracts from the bulb remains limited. Thus, further exploration of metabolite profiling and its pharmacological relevance in Dayak onions is warranted.

Ultra-performance liquid chromatography-mass spectrometry/mass spectrometry (UPLC-MS/MS) is an advanced and precise analytical technique for profiling metabolites in biological samples [12]but they all have disadvantages either in terms of sensitivity or of selectivity. The number of samples that can be analysed, the low volume of samples available during the experiment and the need to identify different degradates are all obstacles that new techniques are able to overcome. The work presented here summarizes progress in the field of metrology as concerns online solid phase extraction technology coupled with liquid chromatography followed by tandem mass spectrometry detection. Recently developed analytical techniques were validated for both 18 pesticides and their degradates and 17 pharmaceuticals and their degradates. Limits of quantification from 20 to 70 ng L−1 for pharmaceuticals and from 15 to 25 ng L−1 for pesticides and metabolites have been obtained, with linearity range up to 1 µg L−1. The limits of quantification of a few nanograms per litre, the possibility of working on less than 1 mL of sample and the simultaneous quantification of the target products and their transformation products are all advantages that are demonstrated by two environmental applications. The first application concerns the evaluation of ecotoxicological effects of pesticides on aquatic organisms exposed in mesocosms. The second application aims to determine the adsorption constants of pharmaceutical molecules on soils and river sediments. For both applications, the robustness, range of linearity and limit of quantification of the developed analytical methods satisfy the requirements for laboratory experiments conducted under controlled conditions. Specific constraints generated by this type of experiment (adding CaCl2 for the adsorption study and filtration of the water coming from the mesocosms. It is extensively applied to determine the biochemical constituents of plant extracts. The extraction process, pivotal in phytochemical analysis, is influenced by the choice of solvent, which determines the quality and quantity of the metabolites extracted [13]. Ethanol and water extracts are commonly used in ethnopharmacological research due to their varied extraction capabilities.

This research aims to carry out a detailed comparative analysis of the metabolite profiles obtained from the *E. bulbosa* bulb extracts using ethanol and water as solvents, utilizing UPLC-MS/MS technology. In addition, the research examines the cytotoxic potential of both extracts on T47D human breast cancer cells. Evaluating cytotoxic activity is essential to correlate with the quantity and quality of bioactive compounds obtained using different solvents. The findings will inform the optimal solvent choice for preparing Dayak onion raw material, maximizing its therapeutic efficacy, and contributing to the development of natural anticancer drugs.

MATERIALS AND METHODS

Plant material

E. bulbosa bulbs were gathered from East Kalimantan in Indonesia. The specimens were cataloged at the UPTD Materia Medika in Malang, under the accession number 074/348/102.7/2021. These plants are currently conserved at the Pharmacognosy Laboratory in the Department of Pharmacy at the Maulana Malik Ibrahim State Islamic University of Malang.

Extraction with 96% ethanol

Fifty grams of the powdered plant material were placed into a measuring cup and liquefied with 500 ml of 96% ethanol, which was divided into two clusters (300 and 200 ml). This mixture was then extracted using the ultrasound-assisted extraction (UAE) method with a sonicator for 3 × 10 minutes. Subsequently, the extract was passed through a filter and subsequently dehydrated using a rotary evaporator followed by an oven [14].

Water extraction

One hundred and twenty-two grams of fresh Dayak onion bulbs were extracted using 610 ml of hot water by maceration method. The extract was then filtered and dried using the freeze-drying method.

Thin layer chromatography (TLC) analysis

A standard solution of 1,4-naphthoquinone and the extracts was prepared at a concentration of 10,000 ppm. Two microliters of the standard solution and extracts were then spotted onto a silica Gel 60 F254 plate. The plate underwent elution with a chloroform:methanol (8:2) solvent mixture. Post-elution, it was subjected to UV visualization at 254 and 366 nm wavelengths. Following this, a 10% sulfuric acid (H_2SO_4) staining solution was applied for derivatization, and the plate was heated to develop visible stain colors, after which it was re-examined under UV light at 366 nm [15].

The analysis of UPLC-QToF-MS

Using UPLC-MS instruments with a QToF detector and positive electrospray ionization for ion generation, the analysis through UPLC-QToF-MS was carried out on an Acquity C18 column with dimensions of 1.8 µm; 2.1 × 150 mm. A mixture of water (of HPLC grade) and formic acid (sourced from Merck, Darmstadt, Germany) was used in a 99.9 to 0.1 [*v*/*v*] ratio. The eluent employed was acetonitrile (provided by Merck, Darmstadt, Germany), used in a 99.9 to 0.1 [*v*/*v*] ratio, within a gradient elution setup. The desolvation temperature was 350°C, while the source temperature was 100°C. Following the solution of a 10 mg extract in an absolute methanol-filled 10 ml volumetric flask, 5 µl volumes were introduced into the UPLC-MS apparatus. Area was expressed as a percentage based on chromatogram data. The study parameters were established using the positive ion mode, and spectra were acquired across a mass range from *m*/*z* 120 to 1,000. Masslynx software version 4.1 (by Waters, Massachusetts, USA) was utilized to process the chromatograms. Component identification was performed by comparing the measured *m*/*z* ratios in Masslynx with

those listed in PubChem (https://pubchem.ncbi.nlm.nih.gov/). Confirmation of a compound's identity was determined through MS/MS fragment comparison and a deviation of less than 5 ppm to ensure accuracy [16].

Cell culture

The T47D breast cancer cell line was procured from Dr. Masashi Kawaichi at the Nara Institute of Science and Technology in Japan. These cells were maintained in a monolayer using high-glucose Dulbecco's Modified Eagle Medium from Gibco, USA, enriched with 10% (*v*/*v*) fetal bovine serum from Sigma, USA, along with 150 IU/ml of penicillin and 150 µg/ml of streptomycin from Gibco, USA, plus 1.25 µg/ml of amphotericin B also from Gibco, USA. They were kept at 37°C in a humidified environment with 5% CO_2 and 100% atmosphere. The T47D cells were utilized for the experiments when they reached 80%–90% confluency [17].

Cell viability assay

The growth rate of T47D cells was assessed via the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Initially, T47D cells (2×10^3 cells/well) were distributed into a 96-well plate and left to attach throughout the night. Post-attachment, the cells were treated for 24 hours with varying agents: 96% ethanol extract of *E. bulbosa*, water extract of *E. bulbosa*, 1,4-Naphthoquinone (from Sigma-Aldrich, USA) at concentrations ranging from 50–500 µM, and doxorubicin (DOX) (from Sigma-Aldrich, USA) at 0.01–10 µM. Cells without treatment served as the negative control. Following the exposure to treatments, each well received 100 µl of MTT solution (0.5 mg/ml, supplied by Biovision) and was incubated for an additional 4 hours at 37°C in an atmosphere of 5% CO_2. The MTT formazan product was then dissolved using an sodium dodecyl sulfate halt agent blended with 0.01 N HCl and left in the dark overnight. The solubilized purple formazan's absorbance was measured at 595 nm using an ELISA plate reader (Corona SH-1000). These treatments were replicated three times to determine the IC50 value—the necessary concentration to inhibit cell growth by 50% relative to the control group [18].

Cell cycle analysis

Flow cytometry was used to conduct cell cycle analysis, employing propidium iodide (PI) for staining. T47D cells were grown to a density of 2×10^5 cells per well in six-well plates. Following treatments with extracts of *E. bulbosa*, 1,4-Naphthoquinone (50–500 µM), and DOX (0.01–10 µM) from Sigma-Aldrich, USA, the culture medium was discarded, and the cells were detached using trypsin, then centrifuged at 2,000 rpm for 3 minutes. The cell harvest was then fixed in ethanol at 4°C for half an hour. Subsequently, the cells were rinsed with cold phosphate-buffered saline (PBS) and centrifuged again under the same conditions. The resulting cell sediment was then reconstituted in a PI mixture (50 µg/ml in PBS with 1% Triton X-100 from Merck) and RNase A devoid of DNase (20 µg/ml), and incubated at 37°C for 30 minutes. The final cell analysis was conducted using a flow cytometer (FACS Calibur, BD Biosciences, USA), where cell debris was electronically excluded, and the red fluorescence intensity was measured using the FL1 channel in logarithmic mode [14].

Apoptosis assay

The apoptosis assay was conducted utilizing the Annexin V-fluorescein isothiocyanate (FITC)/PI staining protocol for flow cytometry after treatments with *E. bulbosa* extract, DOX, and 1,4-naphthoquinone. In summary, after collection, cells were stained for 10 minutes in darkness at ambient temperature using the Annexin-V-FLUOS kit by Roche, which includes 100 µl of binding buffer, 2 µl of Annexin V, and 2 µl of PI. Following staining, the cells were subjected to analysis with a flow cytometer (FACS Calibur, BD Biosciences, USA). FITC was detected by measuring the fluorescence intensity with the FL-1H channel. The proportion of apoptotic cells was then calculated using the Cell Quest software provided by BD Bioscience [19].

RESULTS AND DISCUSSION

Extract yield analysis

In this study, the yield of the 96% ethanol extract was found to be higher (yield percentage: 4.465% ± 0.21%) compared to the yield of the water extract (yield percentage: 4.394% ± 0.52%). The extraction results using 96% ethanol solvent had a higher average than those using water as the solvent. This is presumed to be due to the use of the UAE method which can extract *E. bulbosa* more optimally by using ultrasonic waves compared to the maceration extraction method, which is just a soaking process without the aid of additional forces. Moreover, the drying method used is also believed to affect the results obtained. The freeze-drying method can produce extracts with a very lightweight because all water content in the extract has been optimally dried during the drying process [20]. If the weight of the extract is very light, then the yield value will also be low. Therefore, the extract obtained from water solvent extraction using the freeze-drying method has a lower yield value than the extract obtained from 96% ethanol solvent extraction.

Identification with TLC

The results of the identification using the TLC approach indicated that the standard for 1,4-naphthoquinone was at an retention factor (used in chromatography) (Rf) of 0.90. Stains at the same Rf were also detected in both ethanol and water extracts. The 96% ethanol extract identified stains of 1,4-naphthoquinone, alkaloids, flavonoids, terpenoids, steroids, and tannins. Whereas the water extract showed stains of compounds such as 1,4-naphthoquinone, alkaloids, flavonoids, and steroids.

Metabolite profiling

Metabolite profiling is a method used to analyze compounds with the aim of profiling the metabolite compounds contained in a plant [21]. This instrument was chosen because it has advantages over other analytical tools such as higher resolution which results in more efficient compound separation, smaller column particles leading to higher sensitivity, shorter analysis time due to higher flow rates, the ability to separate

smaller compounds because of higher pressure, and requires less quantity [22].

The choice of solvent in the extraction process is a critical factor due to the differing solubility of compounds. Solvents such as ethanol are often used in herbal extractions because of their ability to extract a wide range of chemical compounds, including both hydrophobic and hydrophilic substances. Meanwhile, water is a more selective solvent and typically only extracts highly polar compounds [23].

This research employed the advanced analytical method of UPLC-QToF-MS/MS for profiling metabolites[12] but they all have disadvantages either in terms of sensitivity or of selectivity. The number of samples that can be analysed, the low volume of samples available during the experiment and the need to identify different degradates are all obstacles that new techniques are able to overcome. The work presented here summarizes progress in the field of metrology as concerns online solid phase extraction technology coupled with liquid chromatography followed by tandem mass spectrometry detection. Recently developed analytical techniques were validated for both 18 pesticides and their degradates and 17 pharmaceuticals and their degradates. Limits of quantification from 20 to 70 ng L−1 for pharmaceuticals and from 15 to 25 ng L−1 for pesticides and metabolites have been obtained, with linearity range up to 1 µg L−1. The limits of quantification of a few nanograms per litre, the possibility of working on less than 1 mL of sample and the simultaneous quantification of the target products and their transformation products are all advantages that are demonstrated by two environmental applications. The first application concerns the evaluation of ecotoxicological effects of pesticides on aquatic organisms exposed in mesocosms. The second application aims to determine the adsorption constants of pharmaceutical molecules on soils and river sediments. For both applications, the robustness, range of linearity and limit of quantification of the developed analytical methods satisfy the requirements for laboratory experiments conducted under controlled conditions. Specific constraints generated by this type of experiment (adding CaCl2 for the adsorption study and filtration of the water coming from the mesocosms. This method enables the detection and measurement of substances with great precision and clarity. The information garnered from the UPLC-QToF-MS/MS apparatus is displayed in the form of chromatograms. The chromatograms produced are shown in Figures 1 and 2.

Based on the metabolite profiling analysis results, it is known that the ethanol extract of *E. bulbosa* contains 32 identified compounds (Table 1). In contrast, the water extract has 27 compounds that have been successfully identified (Table 2). This difference may be attributed to ethanol's efficiency in extracting a greater number of compounds, including nonpolar ones.

Tables 1 and 2 display the compounds detected via UPLC-QToF-MS/MS in both the ethanol extract and the water extract. One factor that may influence the differences in the metabolite profiles is the variance in solvents used. Ethanol and water solvents possess different polarities where ethanol is a semi-polar solvent and water is a polar solvent. Ethanol is considered a universal solvent, thereby capable of extracting the majority of compounds that are both polar and nonpolar in nature from the plant material [24,25]. The percentage levels of transresveratrol, oxyresveratrol, and 1,4-naphthoquinone compounds in the ethanol extract of *E. bulbosa* have been identified in Table 1, at peak numbers 10, 17, and 21, respectively, with percentages of 1.035%, 1.453%, and 1.753% in order. Although the concentrations of these compounds in the extract are not very high, they seem to play a role in anticancer activity.

The analysis of the metabolite profiles revealed a total of 32 compounds from the ethanol extract and 27 from the aqueous extract, with 3 compounds common to both extracts, namely 1,4-naphthoquinone ($C_{10}H_6O_2$), L-lysine sulfate ($C_6H_{14}N_2O_4S$), and Epirizole ($C_{11}H_{14}N_4O_2$). The presence of these compounds in both extracts indicates that they are sufficiently polar to be soluble in water, yet they also possess adequate solubility in ethanol. This data was obtained using a Venn diagram (Fig. 3).

Cytotoxicity test results on breast cancer cell line

In this study, after the identification of phytochemical in the ethanol and water extract of *E. bulbosa*, we proceeded to conduct *in vitro* verification through cytotoxicity testing on the T47D cells. The purpose of this test is to validate the anticancer activity of both extracts.

The cytotoxic test (Fig. 4 and Table 3) indicates that the ethanol extract has low anticancer potential with an IC_{50} value of 202.37 µg/ml. This IC_{50} value refers to the concentration at which the extract can inhibit the growth of 50% of the tested cancer cell population. Meanwhile, the water extract demonstrated no anticanceractivity, with an IC_{50} value of 1,020 µg/ml. According to the National Cancer Institute, an extract is considered active when it exhibits cytotoxic activity with an IC50 value <30 µg/ml. Moderate activity is defined when the IC value is ≥30 µg/ml and the IC50 is <100 µg/ml. The extract is deemed to have low activity or is inactive when the IC50 value is >100 µg/ml [26,27].

Induction of apoptosis and cell cycle arrest

As further evidence from the cytotoxicity assay results showing that compounds in the ethanol extract of *E. bulbosa* have a low inhibitory effect on T47D cells. In this study, further evaluation of the effects of ethanole extract through apoptosis and cell cycle trials was conducted. In addition, the apoptotic activity and the influence of cell cycles were also compared with the chemotherapeutic drugs DOX and the 1,4-naftokuinon compound. On the contrary, the quantitative assessment of cell death, which included the early and late stages of apoptosis, indicated that the use of ethanol extracts of *E. bulbosa* differed significantly in effect when compared to the nontreated control group, as well as with the groups treated with Doxoroubicine and 1,4-naphtokuinon ($p < 0.001$).

In addition to triggering cell mortality, the amplification of anticancer activities may also be achieved by altering the cell cycle. The analysis of cell cycle phases post-treatment was conducted using flow cytometry. T47D cells underwent a 24-hour treatment with 10 nM DOX, the half-maximal inhibitory

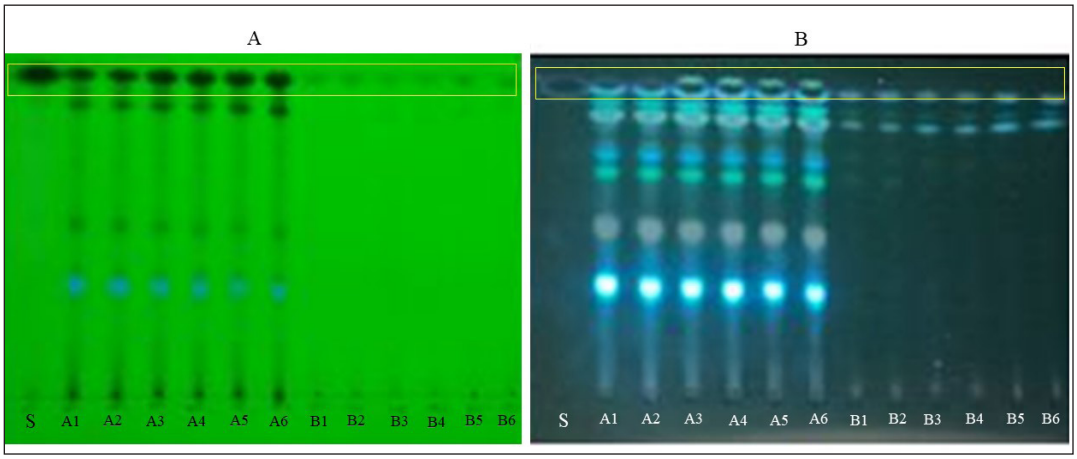

Figure 1. TLC results at UV 254 nm; S = standard 1,4-naphthoquinone; A1–A6 = *Eleutherine bulbosa* ethanol extract; B1–B6 = *Eleutherine bulbosa* water extract (A). UV 366 nm = standard 1,4-naphthoquinone; A1-A6 = *Eleutherine bulbosa* ethanol extract; B1–B6 = *Eleutherine bulbosa* water extract (B).

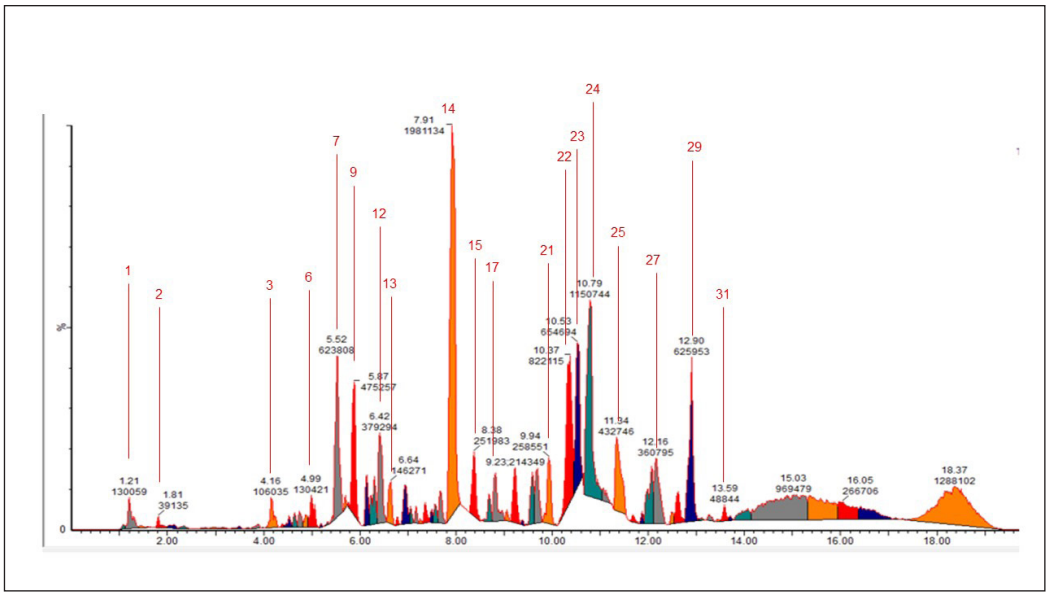

Figure 2. Chromatogram of the 96% ethanol extract of *E. bulbosa*.

concentration (IC50) of *E. bulbosa* ethanol extract, and the IC50 of 1,4-naphthoquinone. Results in Figure 6A and B indicate that there was a substantial impact on the distribution of cell cycle phases of cells treated with *E. bulbosa* ethanol extract ($p < 0.0001$). Administration of this extract resulted in an increase in the sub G1 and S phases, as well as a significant decrease in accumulation in the G2-M and G1 phases. Conversely, the administration of DOX and 1,4-naphthoquinone had no effect on the cell cycle. In Figure 6A and B, it is noted that the cell cycles of DOX and 1,4-naphthoquinone do not differ from the untreated group ($p > 0.0001$).

This study utilized LC-MS/MS techniques to identify chemical components in the ethanolic extract of *E. bulbosa*. According to the results displayed in Table 1, it was determined that the extract contains the compound oxyresveratrol, known for its significant anticancer potential [28].

Previous research has recognized oxyresveratrol as a compound that can affect the expression of genes related to apoptosis (programmed cell death) and cell cycle control, as well as DNA repair in Michigan cancer foundation-7 (a breast cancer cell line) (MCF-7) cells. This effect is crucial in cancer therapy as it can halt the growth of cancer cells and induce death in damaged cells [29].

In addition, studies suggest that oxyresveratrol has the ability to suppress the proliferation of human lung squamous carcinoma cells by inducing arrest in the S-phase of the cell cycle and promoting cell death through apoptosis. The arrest in the S phase prevents cancer cells from continuing DNA replication, an essential step in cell division. Apoptosis induction compels the cell to activate its own death mechanisms [30,31].

In addition to that in Table 1 it has been identified that the ethanolic extract of *E. bulbosa* contains trans resveratrol, in

Table 1. Chromatogram analysis results of LCMS/MS from the 96% ethanol extract of *E. bulbosa.*

No	Rt	% Area	Measured mass	Calculated mass	Formula	Compound name
1	1.211	0.882%	380.0724	380.0719	$C_{21}H_{16}O_5S$	V
2	1.809	0.265%	234.1119	234.1117	$C_{11}H_{14}N_4O_2$	Epirizole
3	4.157	0.719%	645.201	645.2008	$C_{39}H_{35}NO_4S_2$	Methanethiol
4	4.374	0.060%	327.1109	327.1107	$C_{18}H_{17}NO_5$	Tranilast
5	4.642	0.260%	376.1382	376.1383	$C_{17}H_{20}N_4O_6$	Riboflavin
6	4.994	0.884%	483.1474	483.1478	$C_{25}H_{21}N_7O_2S$	2-{[4-Amino-5-(3-phenyl-1H-pyrazol-5-yl)-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(4-phenoxyphenyl)acetamide
7	5.521	4.228%	418.1261	418.1266	$C_{14}H_{30}N_2O_6S_3$	N,N-dimethyl-2-methylsulfonylpent-4-en-1-amine;N-(1-methylsulfonylbut-3-enyl)methanesulfonamide
8	5.697	0.162%	408.1441	408.1443	$C_{22}H_{24}N_4S_2$	1-Isopropyl-3-[(E)-(isopropylimino)(phenyl)methyl]-6-phenyl-1,3,5-triazine-2,4(1H,3H)-dithione
9	5.872	3.221%	244.073	244.0729	$C_6H_{16}N_2O_6S$	l-lysine sulfate
10	6.153	1.035%	228.079	228.0787	$C_{14}H_{12}O_3$	Trans-resveratrol
11	6.308	1.276%	234.0905	234.0906	$C_{14}H_{10}N_4$	3,6-Diphenyl-1,2,4,5-tetrazine
12	6.421	2.571%	596.2134	596.2132	$C_{30}H_{28}N_8O_6$	Methyl N-(4-{[(2,4-diaminopyrido[2,3-d]pyrimidin-6-yl)methyl](formyl)amino}benzoyl)-5-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-L-norvalinate
13	6.638	0.991%	214.1004	214.1004	$C_8H_{20}N_2Cl_2$	1-Isopropyl-4-piperidinamine dihydrochloride
14	7.911	13.429%	256.0734	256.0736	$C_{15}H_{12}O_4$	“Isoliquiritigenin”
15	8.375	1.708%	270.0913	270.0917	$C_8H_{19}N_4O_2SCl$	N-(1-Amino-2-propanyl)-N’-(2-cyanoethyl)-N,N’-dimethylsulfuric diamide hydrochloride
16	8.818	1,422%	268.0740	268.0736	$C_{16}H_{12}O_4$	Formononetin
17	9.233	1.453%	244.0739	244.0736	$C_{14}H_{12}O_4$	Oxyresveratrol
18	9.388	0.075%	284.0689	284.0685	$C_{16}H_{12}O_5$	Olmelin / calycosin
19	9.584	1.153%	258.0885	258.0884	$C_{10}H_{15}N_4O_2Cl$	Morinamide hydrochloride
20	9.697	1.552%	267.0925	267.0929	$C_{13}H_{17}NO_3S$	Rasagiline mesylate
21	9.936	1.753%	158.0848	158.0848	$C_{10}H_6O_2$	1,4-Naphthoquinone
22	10.371	5.572%	298.0789	298.0787	$C_{14}H_{12}O_3$	Oxybenzone
23	10.526	4.438%	228.0786	228.0787	$C_{14}H_{12}O_3$	Oxybenzone”
24	10.794	7.800%	226.0633	226.0630	$C_{14}H_{11}O_3$	Hydroxy(diphenyl)acetate “benzilate”
25	11.869	0.177%	324.1469	324.1471	$C_{31}H_{24}O_8$	3-(4-Biphenylyloxy)-4-oxo-4H-chromen-7-yl 3,4,5-trimethoxybenzoate
26	12.066	1.936%	271.1938	271.1936	$C_{18}H_{25}NO$	(9α,13α,14α)-3-Methoxy-17-methylmorphinan
27	12.158	2.446%	513.4502	513.4506	$C_{29}H_{59}N_3O_4$	4-(2-{Dimethyl[2-(octyloxy)-2-oxoethyl]ammonio}ethyl)-1-methyl-1-[2-(octyloxy)-2-oxoethyl]piperazin-1-ium
28	12.614	0.861%	270.0885	270.0884	$C_{11}H_{15}N_4O_2Cl$	Nitenpyram
29	12.903	4.243%	356.0896	356.0896	$C_{19}H_{16}O_7$	Ethyl 5,7-dihydroxy-3-(4-methoxyphenyl)-4-oxo-4H-chromene-2-carboxylate
30	13.121	0.042%	286.085	286.085	$C_{17}H_{18}S_2$	7,8-Dimethyl-3-phenyl-1,5-dihydro-2,4-benzodithiepine
31	13.430	0.037%	667.4152	667.4152	$C_{33}H_{65}NO_8S_2$	(4S)-4-[2-[2-hydroxy-3-(2-propoxyethoxy)propyl]sulfanylethyl]-2-[[(3S)-1-[2-hydroxy-3-(2-propoxyethoxy)propyl]sulfanyl-4-oxopentan-3-yl]amino]-2,6,6-trimethylheptan-3-one
32	13.711	0.087%	466.3054	466.3056	$C_{26}H_{38}N_6O_2$	N-Benzyl-4-(isobutylamino)-2-[4-(tetrahydro-2H-pyran-3-ylmethyl)-1-piperazinyl]-5-pyrimidinecarboxamide

Table 2. Outcomes of the Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LCMS/MS) chromatogram analysis conducted on the water extract of *E. bulbosa*.

No	Rt	Area %	Measured mass	Calculated mass	Rumus formula	Compound name
1	1.232	3.110%	380.0709	380.0712	$C_{13}H_{20}N_2O_7S_2$	[(3-Methyl-4-nitrosophenyl)imino]di-2,1-ethanediyl dimethanesulfonate
2	1.717	0.329%	234.1121	234.1117	$C_{11}H_{14}N_4O_2$	Epirizole
3	2.089	0.005%	392.2188	392.2186	$C_{19}H_{24}N_{10}$	3-(2-Methyl-2-propanyl)-6-[4-(1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1-piperazinyl][1,2,4]triazolo[4,3-b]pyridazine
4	2.202	0.038%	253.1555	253.1557	$C_{10}H_{24}N_3O_2Cl$	N-{3-[(4-Aminobutyl)amino]propyl}-2-methoxyacetamide hydrochloride (1:1)
5	3.692	0.197%	187.0646	187.0643	$C_5H_{15}N_3Cl_2$	Piperazine
6	4.115	0.172%	418.1439	418.1437	$C_{19}H_{18}N_{10}S$	4-N-ethyl-6-[5-(1-methylpyrazol-4-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl]-4-N-(1,3,4-thiadiazol-2-yl)pyrimidine-2,4-diamine
7	4.291	0.141%	383.1828	383.1824	$C_{20}H_{34}NOBr$	6-(Decylamino)-5,6,7,8-tetrahydro-2-naphthalenol hydrobromide (1:1)
8	4.353	0.115%	531.1727	531.1729	$C_{31}H_{25}N_5O_2S$	2-(1,3-Benzothiazol-2-yl)-1-benzyl-5-[2-(1H-indol-3-yl)ethyl]-4-methyl-1H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione
9	4.487	0.014%	517.1892	517.1890	$C_{18}H_{31}N_9O_5S_2$	Arginylcysteinylcysteinylhistidine
10	4.881	0.056%	629.2309	629.2308	$C_{33}H_{35}N_5O_6S$	2-(1,1-Dioxido-3-oxo-1,2-benzothiazol-2(3H)-yl)-N-{1-(1-methyl-1H-benzimidazol-2-yl)-2-oxo-2-[(tetrahydro-2-furanylmethyl)amino]ethyl}-N-[2-(2-methylphenyl)ethyl]acetamide
11	5.697	0.438%	234.0901	234.0899	$C_6H_{14}N_6O_2S$	N,N-Dimethyl-2-[(1-methyl-1H-tetrazol-5-yl)amino]ethanesulfonamide
12	6.421	0.087%	244.0727	244.0729	$C_6H_{16}N_2O_6S$	l-lysine sulfate
13	7.032	1.266%	244.0754	244.0749	$C_{15}H_8N_4$	1-Phenyl-1H-benzimidazole-5,6-dicarbonitrile
14	7.208	5.627%	256.0768	256.0768	$C_{15}H_{13}N_2Cl$	Chlormidazole atau 1-Benzyl-2-(chloromethyl)-1H-benzimidazole
15	7.764	1.729%	270.0899	270.0899	$C_9H_{14}N_6O_2S$	6-(Dimethylamino)-N,N-dimethyl-7H-purine-2-sulfonamide
16	8.312	2.417%	244.0725	244.0729	$C_6H_{16}N_2O_6S$	l-lysine sulfate
17	8.417	0.282%	242.0589	242.0589	$C_8H_{16}N_2O_2Cl_2$	3-Aminoquinuclidine-3-carboxylic acid dihydrochloride
18	8.530	0.140%	310.0857	310.086	$C_6H_{18}N_2O_{12}$	Nitric acid;propane-1,2,3-triol
19	8.614	1.122%	244.0763	244.0768	$C_{14}H_{13}N_2Cl$	Dibazole
20	8.819	0.014%	314.1156	314.1154	$C_{18}H_{18}O_5$	Flavokawain A
21	8.923	0.328%	258.0909	258.0906	$C_{16}H_{10}N_4$	Ethenetricarbonitrile
22	9.254	1.131%	298.0843	298.0841	$C_{17}H_{14}O_5$	Coumafuryl
23	9.844	0.568%	158.0848	158.0848	$C_{10}H_6O_2$	1,4-Naphthoquinone
24	10.070	7.448%	244.0745	244.0746	$C_8H_{18}N_2O_2Cl_2$	Dimethyl hexanediimidate dihydrochloride
25	11.405	0.299%	305.2836	305.2831	$C_{19}H_{35}N_3$	N-[3-(Dimethylamino)propyl]-N',N'-dimethyl-N-(3-phenylpropyl)-1,3-propanediamine
26	12.221	0.515%	356.0904	356.0903	$C_{12}H_{16}N_6O_5S$	N-[(4,6-Dimethoxy-2-pyrimidinyl)carbamoyl]-1,4-dimethyl-1H-pyrazole-5-sulfonamide
27	12.439	1.128%	288.1010	288.1011	$C_{17}H_{12}N_4O$	5-[2-(2-Naphthyloxy)phenyl]-1H-tetrazole

previous studies it had been that trans-resveratrole is capable of inducing apoptosis in breast cancer cells MCF-7. The process involves activating the path Mitogen-activated protein kinases, which include c-Jun N-terminal kinase and p38 mitogen-activated protein kinase[32].

Therefore, these findings provide additional evidence to support the role of oxyresveratrol and trans resveratrol as active components in *E. bulbosa* extract that may contribute to the observed anticancer activity.

In vitro validation results regarding the anticancer activity of *E. bulbosa* ethanolic extract show significant induction of apoptosis, and strong potential in upregulating the cell cycle through an increase in the sub-G1 and S phases, along with a significant decrease in accumulation in the G2-M and G1 phases [33]cyclins D1 and E and the cyclin-dependent kinase inhibitors p21 (Waf1/Cip1.

These findings concur with earlier studies which found that the ethanol extract of *E. bulbosa* hindered the growth of retinoblastoma cancer cells, demonstrating a half-maximal inhibitory concentration (IC50) of 15.7 µg/ml. Cells showed initial indications of apoptosis and increased regulation of the cell cycle [34].

Genetically, the data shows a heightened expression of genes that advance apoptosis (like Bax, p53, and Caspases) and a reduced expression of genes that deter apoptosis (such as Bcl-2 and Nrf-2) [34]. The p53 gene is an important marker in controlling DNA quality and triggers apoptosis in the event of DNA damage [35]. Caspases are a family of proteins that

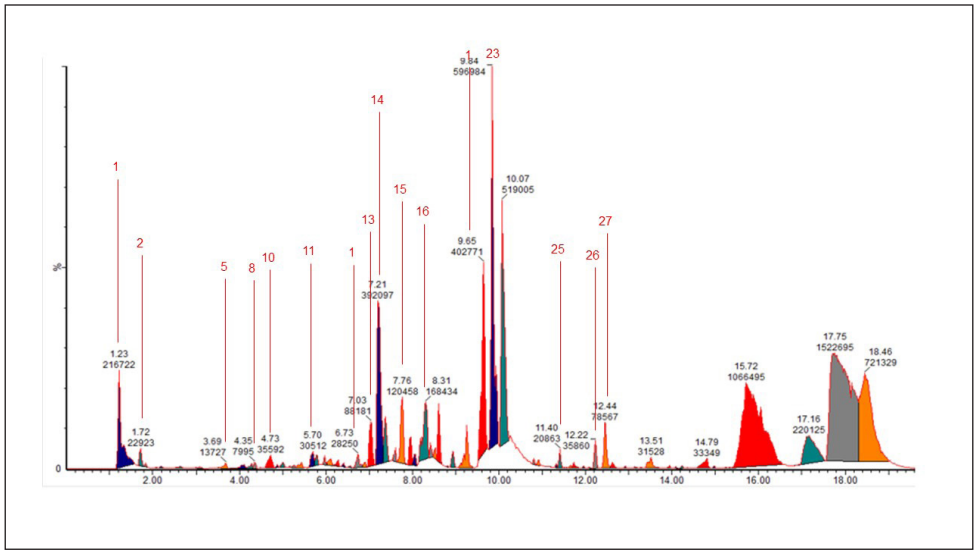

Figure 3. Chromatogram of the water extract of *E. bulbosa*.

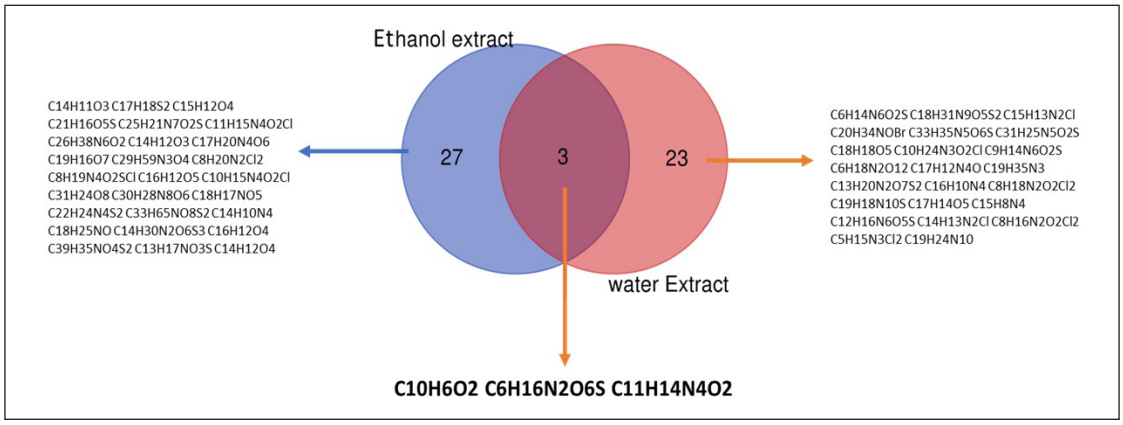

Figure 4. Venn diagram illustrating the compound profiles for ethanol and water extracts of *E. bulbosa*.

Table 3. IC_{50} value (50% inhibitory concentration) of *E. bulbosa* extract against cell line T47D.

	Sample	IC50 ± SD	Category
A	Ethanol extract	202.37 ± 2.29	Low
B	Water extract	1,020.30 ± 10.15	no activity
C	1. 4 naphthoquinone	10.54 ± 0.21	Very potential
D	DOX	115.1 ± 0.54	Low

function in the execution of apoptosis [36]. On the other hand, the Bcl-2 and Nrf-2 genes are involved in the cell's defense mechanisms to prevent apoptosis and oxidative stress [37].

In summary, the findings of this research verify that the ethanolic extract of *E. bulbosa*, which includes oxyresveratrol, is noted to exert a considerable effect on the proliferation of cancer cells [28–31]. Using LC-MS/MS techniques, this study successfully identified this compound and linked it to anticancer activity *in vitro*. In addition, there is evidence that oxyresveratrol contributes to the halting of the cell cycle and the induction of apoptosis, including changes in the expression of genes associated with the apoptosis process.

1,4-naphthoquinone exhibits a higher level of cytotoxic activity compared to the ethanol extract (Table 3), but it does not have an impact on apoptosis and the cell cycle (Fig. 5). This suggests that 1,4-naphthoquinone may have potential as an anticancer agent, but not through the pathways of apoptosis and the cell cycle. In previous studies, it has been reported that 1,4-naphthoquinone exhibits highly potent anticancer activity in several cell lines, including HepG2, HuCCA-1, A549, and MOLT-3. In this study, it was found that 1,4-naphthoquinone has a high-potential IC50; however, in apoptosis and cell cycle tests, it did not show any effects. This may be due to the compound working as an anticancer agent, not through the induction of apoptosis and the cell cycle, but possibly through other mechanisms. Previous research has reported that quinone-based drugs function as anticancer agents through the production of reactive oxygen species. In addition, the cytotoxic effects of clinically used quinone-based anticancer drugs are closely

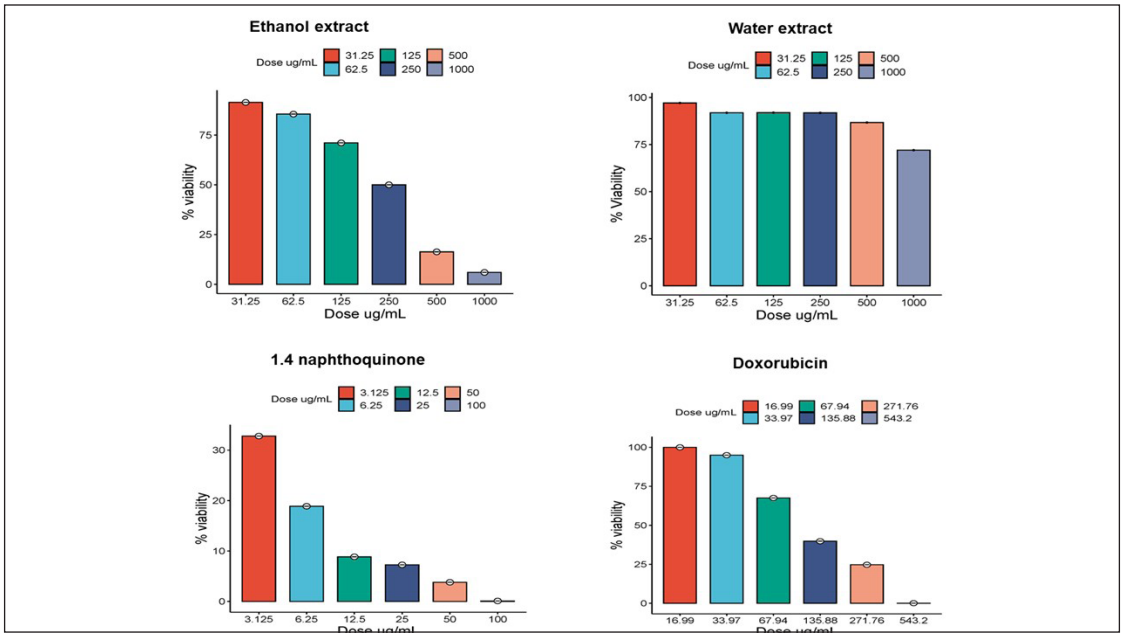

Figure 5. T47D cell viability after treatment with ethanol extract of *E. bulbosa*, water extract, DOX, and 1,4-naphthoquinone. Over a 24-hour period, test samples at different concentrations were administered to T47D cells, and the MTT assay was used to determine cell viability. The profile of cell survival is reported as the average ± SD from three separate trials.

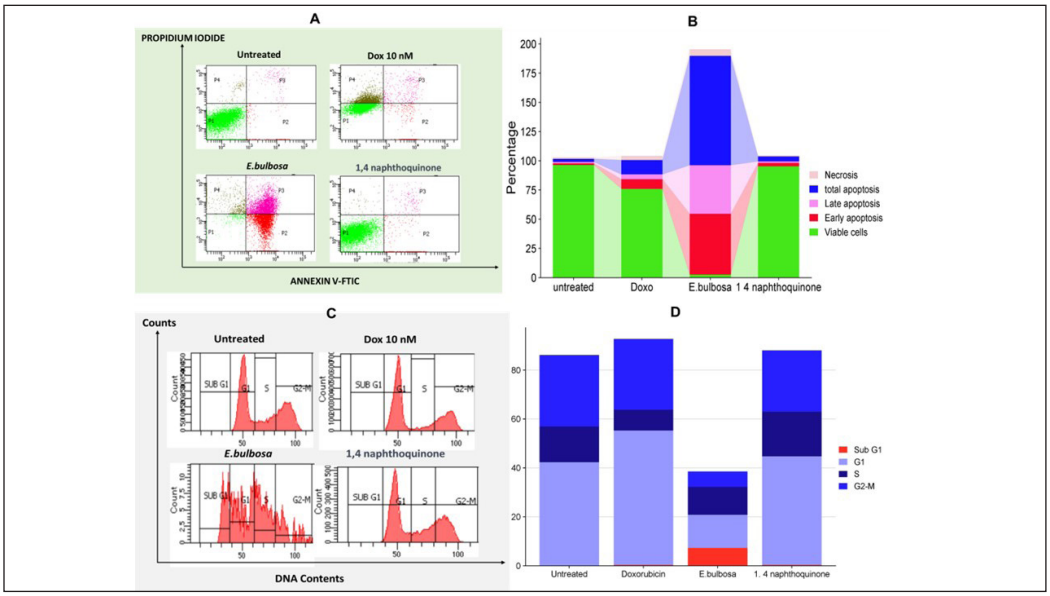

Figure 6. Triggering cell death and altering cell cycle progression in T47D cells of breast cancer by treatment with *E. bulbosa* ethanol extract, DOX, and 1,4-naphthoquinone. Cells were treated with *E. bulbosa* ethanol extract, DOX, and 1,4-naphthoquinone for 24 hours. Further examination assessed the proportion of cell mortality (Fig. A and B) and the allocation of cells in various stages of the cell cycle (Fig. C and D), using flow cytometry after labeling with Annexin V-FITC/PI and PI, correspondingly. The lines indicate the average ± SD from three separate studies. A *p*-value less than 0.001 (calculated through a *post-hoc* LSD test among the groups) was deemed to reflect statistical significance.

related to the inhibition of the DNA topoisomerase II enzyme [38]. DNA topoisomerase II is a crucial enzyme required for DNA replication, chromosome condensation, and chromosome segregation [39]. The DNA opening process is a crucial step where the double-stranded DNA helix needs to be unwound to allow the continuation of the DNA replication process [40].

However, this study is still limited to the use of one type of cancer cell and has not yet deeply explored the specific molecular mechanisms of oxyresveratrol. In addition, *in vitro* results may not fully reflect the same effects within living organisms due to the complexity of interactions within biological systems. Therefore, future research should lead to *in*

vivo studies to evaluate the effectiveness and safety of the *E. bulbosa* ethanolic extract and oxyresveratrol in animal models. Further research should also consider testing against various types of cancer cells to expand understanding of the anticancer activity spectrum of this compound. In addition, research that delves deeper into the molecular mechanisms and signaling pathways involved in the anticancer effects of oxyresveratrol will provide valuable insights for the development of more effective cancer therapies.

CONCLUSION

The metabolite profile obtained from the ethanol extract of *E. bulbosa* revealed the presence of 32 compounds, while its water extract disclosed the presence of 27 compounds. Venn diagram analysis identified three compounds present in both extracts: 1,4-Naphthoquinone ($C_{10}H_6O_2$), l-lysine sulfate ($C_6H_{16}N_2O_6S$), and Epirizole ($C_{11}H_{14}N_4O_2$). In cytotoxic tests, the ethanol extract of *E. bulbosa* demonstrated low anticancer ability with an inhibitory concentration of 50% (IC_{50}) of 202.37 µg/ml. On the other hand, the water extract did not exhibit anticancer effects with an IC50 of 1,020 µg/ml. Despite its low activity, the ethanol extract of *E. bulbosa* is effective in inducing apoptosis and potentially influencing the life cycle of T47D cancer cells. Therefore, this finding suggests that ethanol is a more suitable solvent for extracting bioactive components from *E. bulbosa* that are beneficial in the development of anticancer therapy.

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AUTHOR CONTRIBUTIONS

All authors have made an equal contribution to the writing of this article.

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CONFLICTS OF INTEREST

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

ETHICAL APPROVALS

This research was approved by the Research Ethics Committee on Health of the Faculty of Medicine and Health Science at UIN Maulana Malik Ibrahim Malang with the number: No.139/EC/KEPK-FKIK/2022.

DATA AVAILABILITY

All data generated and analyzed that are included in this research article are available upon request.

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