

# Cytotoxic and Apoptosis-Associated Activity of *Schefflera elliptica* (Blume) Harms Ethanolic Extract in T47D Breast Cancer Cells Supported by LC-HRMS Profiling and Molecular Docking

Made Dharmesti Wijaya<sup>1\*</sup>, Anak Agung Gede Indraningrat<sup>2</sup>,  
Wirdatun Nafisah<sup>3</sup> and Nora Ertanti<sup>4</sup>

<sup>1</sup>Department of Pharmacology, Faculty of Medicine and Health Sciences,  
Warmadewa University, Jl Terompong No. 24, Denpasar-Bali, Indonesia.

<sup>2</sup>Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences,  
Warmadewa University, Jl Terompong No. 24, Denpasar-Bali, Indonesia.

<sup>3</sup>Department of Biology, Faculty of Mathematics and Natural Sciences,  
State University of Surabaya, Jl. Ketintang Gedung D1, Surabaya-Jawa Timur, Indonesia.

<sup>4</sup>Stem Cell Research and Development Center, Airlangga University,  
Jl. Mulyorejo Kampus C, Surabaya-Jawa Timur, Indonesia.

\*Corresponding Author E-mail: dharmestiwijaya@warmadewa.ac.id

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Breast cancer continues to impose a substantial global health burden, and natural products remain an important source of candidates for anticancer research. This study investigated the cytotoxic and apoptosis-associated effects of the ethanolic extract of *Schefflera elliptica* against T47D human breast cancer cells. Cytotoxicity was evaluated using the MTT assay, while apoptosis-associated responses were assessed by Annexin V-FITC/propidium iodide flow cytometry and caspase-3 and caspase-8 immunofluorescence. Metabolite profiling was performed using liquid chromatography–high-resolution mass spectrometry (LC-HRMS), followed by exploratory molecular docking of selected metabolites against the anti-apoptotic protein Bcl-2. The crude ethanolic extract exhibited moderate cytotoxic activity, with an IC<sub>50</sub> of 339 µg/mL. Treatment increased the Annexin V-positive cell population, with significant increases observed at 160 and 320 µg/mL, and was accompanied by increased caspase-3 and caspase-8 immunofluorescence signals. LC-HRMS profiling yielded 39 putatively annotated metabolites, with choline, L-norleucine, and valine among the most abundant. Molecular docking predicted interactions of these metabolites within the Bcl-2 binding pocket, although their predicted binding affinities were weaker than those of venetoclax. Overall, the findings indicate that *S. elliptica* ethanolic extract exhibits moderate cytotoxic activity accompanied by apoptosis-associated cellular responses in T47D cells. The integrated biological, metabolomic, and computational findings provide a basis for further bioactivity-guided investigation of the active constituents and their underlying mechanisms.

**Keywords:** Apoptosis, Breast Cancer, Cytotoxicity, LC-HRMS, Molecular Docking, *Schefflera elliptica*.

Breast cancer continues to rank among the most frequently diagnosed malignancies in women worldwide and remains a substantial global public

health burden. According to data from the Global Burden of Disease (GBD) study and recent global cancer statistics, breast cancer accounts for more

than two million new cases annually. It continues to be a major contributor to cancer mortality among women worldwide.<sup>1-3</sup> Despite significant progress in therapeutic strategies such as surgery, chemotherapy, and radiotherapy, the effectiveness of these treatments is frequently compromised by severe side effects, systemic toxicity, therapeutic resistance, and long-term off-target effects, including anthracycline-induced cardiotoxicity.<sup>4-8</sup> These limitations have encouraged the exploration of alternative or complementary therapeutic agents with improved safety and efficacy profiles.

Natural products derived from medicinal plants have attracted increasing attention as promising sources of anticancer compounds due to their chemical diversity and capacity to modulate various cellular pathways involved in tumor progression.<sup>9-11</sup> Numerous phytochemicals have been reported to suppress tumor cell growth while inducing programmed cell death through apoptosis-mediated mechanisms.<sup>9</sup> Apoptosis is a highly coordinated physiological mechanism responsible for removing damaged or malignant cells, and its dysregulation is a hallmark of cancer progression.<sup>12</sup> In breast cancer cells, apoptosis is commonly regulated by the activation of caspase proteins and the dynamic equilibrium between pro-apoptotic and anti-apoptotic proteins within the Bcl-2 family, which control mitochondrial membrane permeability and downstream caspase activation.<sup>12, 13</sup> Therefore, targeting apoptosis-related pathways, including caspase activation and Bcl-2 inhibition, has become an important strategy in the development of new anticancer agents.

Recent studies investigating plant-derived anticancer compounds frequently integrate *in vitro* cytotoxicity assays with metabolite profiling and computational analysis to better understand their biological activities and potential molecular targets.<sup>14, 15</sup> This integrated strategy is particularly valuable because experimental assays provide direct evidence of cytotoxic or apoptosis-associated activity. At the same time, analytical and computational approaches may help identify candidate metabolites and predict their interactions with apoptosis-related proteins. Related studies have likewise combined experimental bioactivity assessment with chemical profiling and computational approaches to support the characterization of biologically active natural

products and their potential molecular interactions.<sup>16, 17</sup> *Schefflera elliptica* (Blume) Harms, locally known as *kayu tulak*, is an Indonesian medicinal plant traditionally used for therapeutic purposes. Previous studies have reported its antibacterial and antioxidant activities. Phytochemical investigations revealed the presence of bioactive constituents, including triterpenoid-related compounds.<sup>18, 19</sup> However, studies evaluating the cytotoxic and apoptosis-associated activity of *S. elliptica* against breast cancer cells remain scarce, and the relationship between its metabolite profile and potential molecular targets involved in apoptosis has not been comprehensively investigated. T47D cells were selected as an established hormone-responsive human breast cancer model frequently used in cytotoxicity studies of natural products.<sup>20</sup>

Therefore, this research focuses on evaluating the cytotoxic and apoptosis-associated activity of *S. elliptica* ethanolic extract against T47D breast cancer cells while simultaneously characterizing its metabolite composition and potential molecular interactions with apoptosis-related proteins. The objectives of this research were to (i) determine the cytotoxic activity of *S. elliptica* extract against T47D breast cancer cells, (ii) assess apoptosis-associated effects through Annexin V and caspase-based assays, (iii) identify major metabolites using LC-HRMS profiling, and (iv) evaluate the potential interaction of selected compounds with the anti-apoptotic Bcl-2 protein through molecular docking analysis.

## MATERIALS AND METHODS

### Materials

*Schefflera elliptica* leaves were harvested in February 2024 in Gerih Village, Bali, Indonesia. Plant identification was conducted and voucher specimens (fresh and dried) were deposited at the Plant Characterization Laboratory of the “Eka Karya” Bali Botanical Garden, National Research and Innovation Agency (BRIN), Candikuning, Tabanan, Bali, Indonesia, and documented under sample code 1617-31996-1. The T47D human breast cancer cell line (HTB-133) was used in this study. Cells were cultured using Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution. Ethanol

(96%, pro analysis grade) and dimethyl sulfoxide (DMSO) were obtained from Merck Life Science, Darmstadt, Germany. The MTT reagent, Annexin V apoptosis detection kit, and antibodies for caspase-3 and caspase-8 were used for apoptosis analysis. LC/HRMS profiling was carried out on a Dionex Ultimate 3000 RSLCnano HPLC system connected to a Q Exactive High Resolution Mass Spectrometer (Thermo Scientific, USA) equipped with a Hypersil GOLD PFP analytical column (50 × 1 mm, 1.9 µm). Molecular docking analysis was performed using AutoDock Vina implemented in PyRx, and interaction visualization was conducted using Biovia Discovery Studio.

#### **Preparation of Plant Extract**

Fresh *S. elliptica* leaves were sorted to remove damaged and insect-infected material, washed thoroughly with running water, and oven-dried at 40 °C. The dried leaves were then pulverized and passed through a 60-mesh sieve. Subsequently, 100 g of the powdered sample was macerated in 96% ethanol at a solvent-to-sample ratio of 5:1 (v/w) for 24 h, with occasional stirring. The maceration was repeated once under identical conditions. The resulting filtrates were pooled and concentrated using a rotary evaporator at 40 °C to obtain the crude ethanolic extract.

#### **Cytotoxicity Assay (MTT)**

T47D breast cancer cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Cells were subcultured when they reached approximately 70–80% confluence and subsequently used in the cytotoxicity assay. For the MTT assay, cells were plated in 96-well plates at a density of  $1 \times 10^4$  cells/well and allowed to attach for 24 h. A medium blank and an untreated cell control were included for background correction and calculation of relative cell viability, respectively. The cultures were subsequently exposed to *S. elliptica* ethanolic extract at concentrations of 40, 80, 160, 320, and 640 µg/mL for 24 h. Following the exposure period, MTT reagent was added to each well, and the plates were incubated at 37 °C for 3 h to allow formazan crystals to form. The supernatant was carefully discarded, after which the crystals were solubilized with dimethyl sulfoxide. Absorbance was recorded using a microplate reader at 590 nm. Cell viability was expressed as a percentage relative to untreated control cells, and the IC<sub>50</sub> value was calculated from the corresponding dose–response curve.

All experimental conditions were analyzed in three wells within a single experiment (technical triplicates).

#### **Apoptosis Analysis**

Apoptosis was assessed by Annexin V-FITC/propidium iodide (PI) staining and caspase immunofluorescence assays. For Annexin V analysis, T47D cells were seeded in 6-well plates and exposed to *S. elliptica* extract at concentrations of 80, 160, and 320 µg/mL for 24 h. Following treatment, cells were collected, rinsed with buffer, and stained with Annexin V-FITC and PI according to the manufacturer's protocol in the dark before flow cytometric analysis. For immunofluorescence analysis, T47D cells were grown on microslides placed in 24-well plates and treated under identical experimental conditions. After fixation and permeabilization, the cells were incubated with FITC-conjugated antibodies against caspase-3 or caspase-8. Fluorescence signals were subsequently examined using a fluorescence microscope.

Quantification of immunofluorescence signals was performed using ImageJ software. Mean fluorescence intensity (MFI) of the caspase-3 and caspase-8 signals was measured within consistently defined regions of interest (ROIs) across the experimental groups. Background fluorescence was subtracted from the measured fluorescence intensity, and the resulting MFI values were used to quantify relative caspase-3 and caspase-8 immunofluorescence signals. The same image acquisition and analysis settings were applied across all experimental groups.

#### **LC/HRMS Analysis**

The metabolite profile of *S. elliptica* ethanolic extract was characterized by Liquid Chromatography coupled to High Resolution Mass Spectrometry (LC/HRMS). Before analysis, the extract was diluted in a polar solvent, vortex-mixed, centrifuged, and passed through a 0.22 µm syringe membrane filter. Chromatographic separation was achieved using a binary mobile phase comprising solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), with the column maintained at 30°C. Mass spectrometric data were acquired in positive electrospray ionization mode using full MS and data-dependent MS<sup>2</sup> acquisition. Compound annotation was performed using Compound Discoverer software and the mzCloud MS/MS library. Peaks corresponding to identical

compounds were consolidated by selecting the highest intensity signal, and compounds without reliable library matches were excluded from further analysis.

### Molecular Docking Analysis

Molecular docking analysis was conducted to examine the potential binding of major metabolites putatively annotated by LC/HRMS to the anti-apoptotic Bcl-2 protein. The three most abundant compounds with available 3D structures, namely choline, L-norleucine, and valine, were selected as representative ligands for exploratory docking analysis. The three-dimensional structures of the ligands were downloaded from the PubChem database, whereas the crystal structure of the Bcl-2-venetoclax complex (PDB ID: 6O0K), originally reported by Birkinshaw *et al.* (2019),<sup>21</sup> was retrieved from the Protein Data Bank. Venetoclax served as the reference ligand. Docking calculations were performed using AutoDock Vina integrated into PyRx platform. The docking search space was defined around the venetoclax-binding pocket of the Bcl-2 protein. Docking outcomes

were evaluated based on binding affinity scores and ligand-protein interaction residues, while molecular interactions were visualized using Biovia Discovery Studio.<sup>22</sup>

### Statistical Analysis

Experimental data are expressed as the mean  $\pm$  standard deviation (SD). The IC<sub>50</sub> value was estimated from the concentration–response curve generated from the MTT viability assay relative to the untreated control cells. Statistical analysis of apoptosis and caspase immunofluorescence data was conducted using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for pairwise comparisons, using IBM SPSS Statistics version 26. A p-value of less than 0.05 was considered to indicate statistical significance.

## RESULTS

### Cytotoxic activity of *S. elliptica* extract on T47D cells

The ethanolic extract of *S. elliptica* exhibited dose-dependent cytotoxic activity against T47D breast cancer cells. As shown in Table 1, cell viability remained high at 40  $\mu$ g/mL (99.3%) but decreased progressively at higher concentrations, reaching 81.1% at 80  $\mu$ g/mL and 79.3% at 160  $\mu$ g/mL. A more pronounced reduction occurred at 320  $\mu$ g/mL (44.8%), while only 10.2% of cells remained viable at 640  $\mu$ g/mL. The IC<sub>50</sub> value calculated from the dose-response curve was 339  $\mu$ g/mL.

### Apoptosis induction in T47D cells

To examine whether the reduction in cell viability was associated with apoptotic cell death,

**Table 1.** Effects of *S. elliptica* ethanolic extract on the viability of T47D breast cancer cells. Results are presented as mean  $\pm$  SD (n = 3)

| Concentrations ( $\mu$ g/mL) | Cell Viability (%) |
|------------------------------|--------------------|
| 0 (control)                  | 100.0 $\pm$ 9.7    |
| 40                           | 99.3 $\pm$ 6.8     |
| 80                           | 81.1 $\pm$ 3.0     |
| 160                          | 79.3 $\pm$ 4.9     |
| 320                          | 44.8 $\pm$ 6.0     |
| 640                          | 10.2 $\pm$ 0.6     |

**Table 2.** Most abundant compounds putatively annotated from *S. elliptica* ethanolic extract by LC/HRMS

| No | Compound           | Formula                                        | MW (Da) | RT (min) | Peak Area |
|----|--------------------|------------------------------------------------|---------|----------|-----------|
| 1  | Choline            | C <sub>5</sub> H <sub>13</sub> NO              | 103.10  | 1.44     | 1.08E+10  |
| 2  | Dipropylene glycol | C <sub>6</sub> H <sub>14</sub> O <sub>3</sub>  | 134.09  | 0.93     | 5.25E+09  |
| 3  | L-Norleucine       | C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub> | 131.09  | 1.27     | 2.37E+09  |
| 4  | Valine             | C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> | 117.08  | 1.27     | 1.03E+09  |
| 5  | DEET               | C <sub>12</sub> H <sub>17</sub> NO             | 191.13  | 1.04     | 9.50E+08  |
| 6  | L-Valine           | C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> | 117.08  | 1.14     | 5.29E+08  |
| 7  | Isoleucine         | C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub> | 131.09  | 1.57     | 3.78E+08  |
| 8  | Lactamide          | C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub>  | 89.05   | 1.26     | 3.10E+08  |
| 9  | Adenine            | C <sub>5</sub> H <sub>5</sub> N <sub>5</sub>   | 135.05  | 1.30     | 2.61E+08  |
| 10 | $\beta$ -Alanin    | C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub>  | 89.05   | 1.14     | 1.57E+08  |

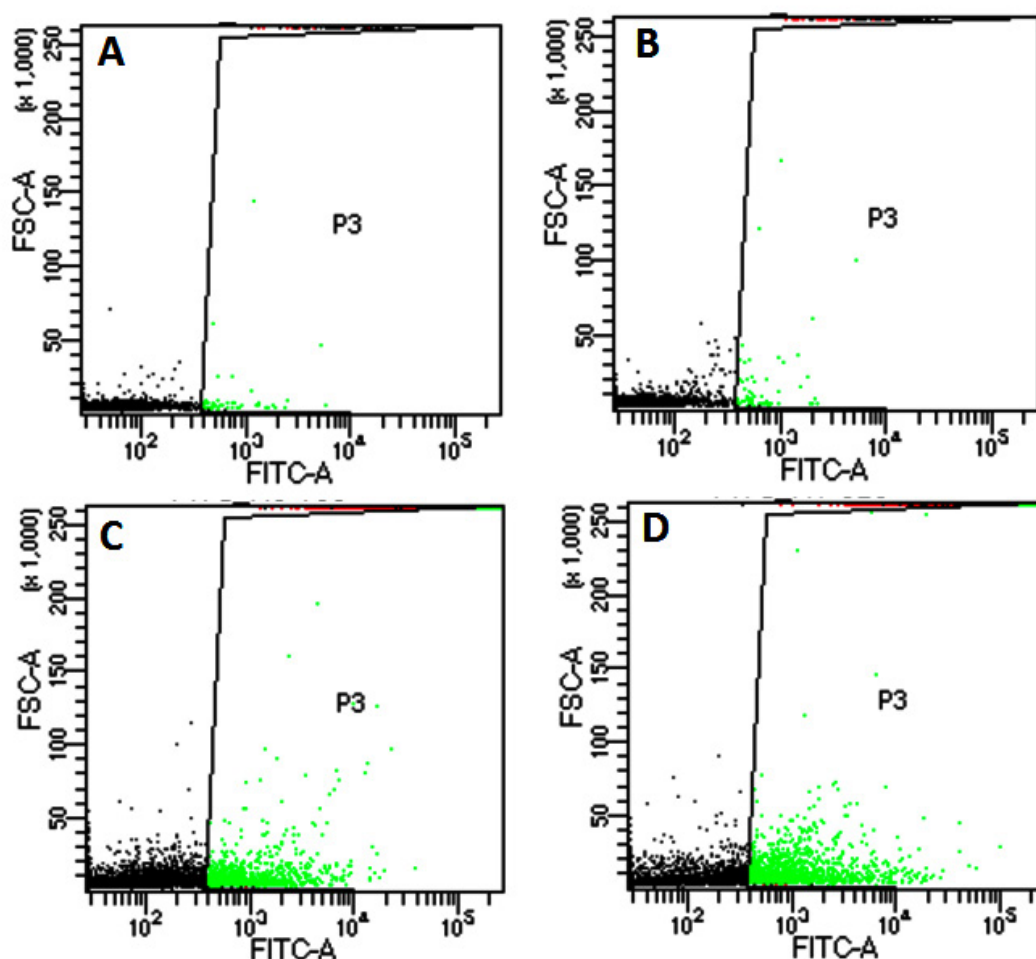
Annexin V-FITC/PI flow cytometric analysis was performed. As shown in Figure 1, exposure to the extract at 80, 160, and 320  $\mu\text{g/mL}$  produced a concentration-dependent increase in the Annexin V-positive cell population relative to the untreated group. Quantification of Annexin V-positive cells (Figure 2) confirmed that apoptosis increased significantly at 160 and 320  $\mu\text{g/mL}$ , while the 80  $\mu\text{g/mL}$  treatment did not differ significantly from the control.

Further assessment of apoptosis-associated responses was performed using caspase immunofluorescence analysis. Caspase-3 immunofluorescence signals were significantly

increased in all treated groups compared with the control (Figure 3). In contrast, caspase-8 immunofluorescence signals showed a more concentration-dependent pattern, with a significant increase observed only at 320  $\mu\text{g/mL}$  (Figure 4).

#### LC-HRMS Metabolite Profiling

LC-HRMS analysis revealed a chemically diverse composition of the *S. elliptica* ethanolic extract, with 39 detected compounds, as illustrated in the chromatographic profile (Figure 5). Among these, choline, L-norleucine, and valine were putatively annotated as the most abundant metabolites (Table 2).



**Fig. 1.** Representative flow cytometry plots of T47D cells treated with *S. elliptica* ethanolic extract and analyzed using Annexin V-FITC/PI staining. The gated Annexin V-positive population was used for quantification of apoptotic cells. (A) Control, (B) 80  $\mu\text{g/mL}$ , (C) 160  $\mu\text{g/mL}$ , and (D) 320  $\mu\text{g/mL}$ .

### Molecular docking analysis

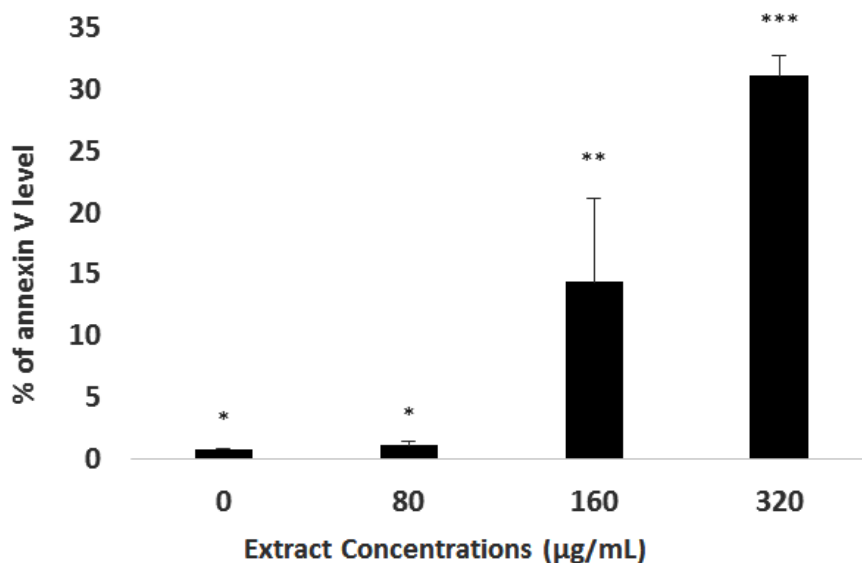
Molecular docking analysis was performed to examine potential binding interactions between selected metabolites putatively annotated in the extract and the anti-apoptotic Bcl-2 protein. Among the tested ligands, venetoclax exhibited the strongest binding affinity (-7.4 kcal/mol), consistent with its established role as a clinically approved Bcl-2 inhibitor. The extract-derived metabolites showed lower binding affinities, with L-norleucine (-4.5 kcal/mol), valine (-4.3 kcal/mol), and choline (-3.2 kcal/mol), as summarized in Table 3. Despite these weaker affinities, interaction analysis revealed that several residues involved in ligand binding—including ASP103, ALA100, TYR202, and VAL148—were shared among multiple ligands (Figure 6).

### DISCUSSION

The present study demonstrated that the ethanolic extract of *Schefflera elliptica* exerts concentration-dependent cytotoxic activity against T47D breast cancer cells, with an  $IC_{50}$  value of

339  $\mu\text{g/mL}$ . According to commonly accepted criteria for crude plant extracts,  $IC_{50}$  values ranging from approximately 100–500  $\mu\text{g/mL}$  are generally classified as moderate cytotoxic activity, whereas values below 100  $\mu\text{g/mL}$  indicate strong cytotoxicity.<sup>23-25</sup> Although the cytotoxic potency observed in the present study is moderate, these findings indicate that *S. elliptica* contains bioactive constituents capable of suppressing breast cancer cell viability. Similar levels of activity have frequently been reported for crude medicinal plant extracts before purification of their active constituents, suggesting that further fractionation may reveal compounds with greater biological potency.<sup>25</sup>

Annexin V flow cytometry demonstrated a concentration-dependent increase in apoptotic cell populations following treatment with the extract. These findings indicate that the reduction in cell viability was accompanied by an apoptosis-associated response. Apoptosis is a tightly regulated physiological process that eliminates damaged or malignant cells while minimizing inflammatory responses, making it one of the most



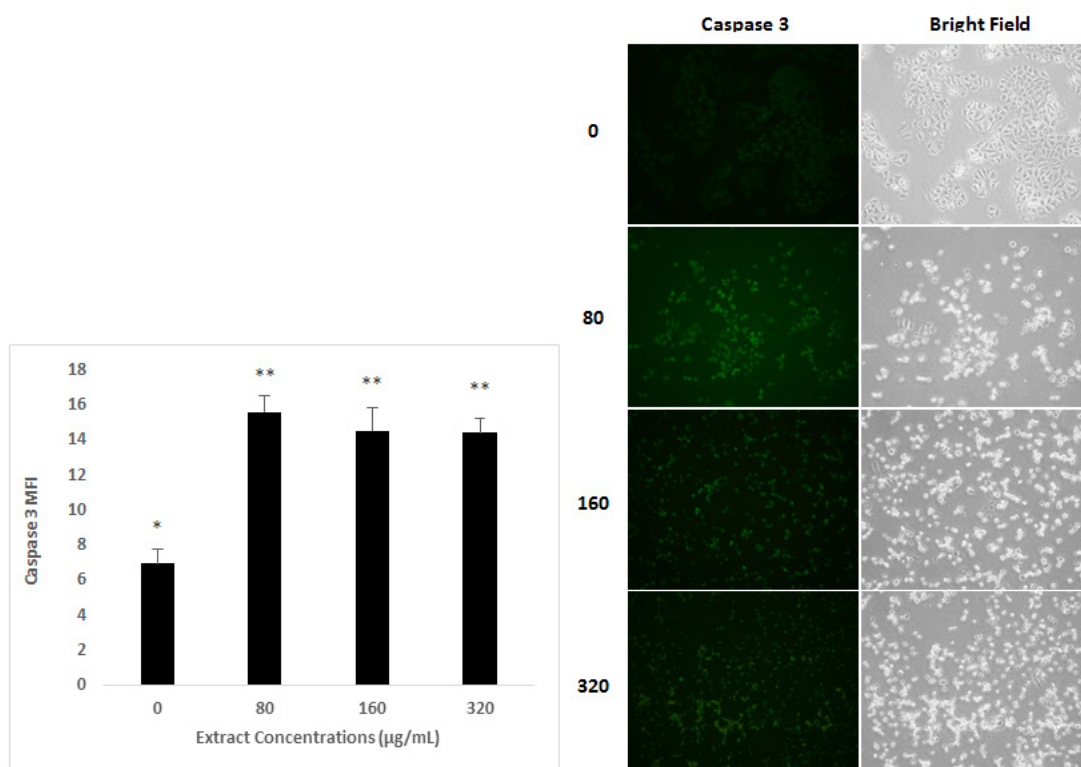
**Fig. 2.** Percentage of Annexin V-positive T47D cells following exposure to *S. elliptica* ethanolic extract at concentrations of 0, 80, 160, and 320  $\mu\text{g/mL}$ . Results are expressed as mean  $\pm$  SD ( $n = 3$ ). Annexin V-positive cells were quantified as the apoptotic cell population. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range test. No significant difference was observed between the control and 80  $\mu\text{g/mL}$  groups. In contrast, treatment with 160 and 320  $\mu\text{g/mL}$  resulted in significantly higher apoptotic cell populations than those in both the control and 80  $\mu\text{g/mL}$  groups ( $p < 0.05$ ). Different symbols indicate statistically significant differences between treatment groups.

desirable mechanisms of action for anticancer agents.<sup>26</sup> Because evasion of apoptosis is a hallmark of cancer progression, restoration of apoptotic signaling has become a major therapeutic strategy in cancer treatment.<sup>27, 28</sup> The dose-dependent increase in Annexin V-positive cells observed in this study therefore supports an association between *S. elliptica* extract treatment and apoptotic cell death in T47D cells.

The apoptosis-associated response was further supported by immunofluorescence analysis of caspase proteins. Caspase-3 immunoreactivity increased significantly in all treated groups relative to untreated cells. The significant increase in caspase-3 immunoreactivity at 80 µg/mL, despite the absence of a significant increase in Annexin V-positive cells at this concentration,

may reflect differences in the sensitivity and biological endpoints measured by these two assays. As a principal executioner caspase, caspase-3 participates in the proteolytic cleavage of numerous intracellular substrates associated with the characteristic morphological and biochemical features of apoptosis.<sup>29, 30</sup> The increased caspase-3 immunoreactivity observed following extract treatment therefore provides additional evidence of an apoptosis-associated cellular response, although the present assay does not distinguish between total and cleaved caspase-3.

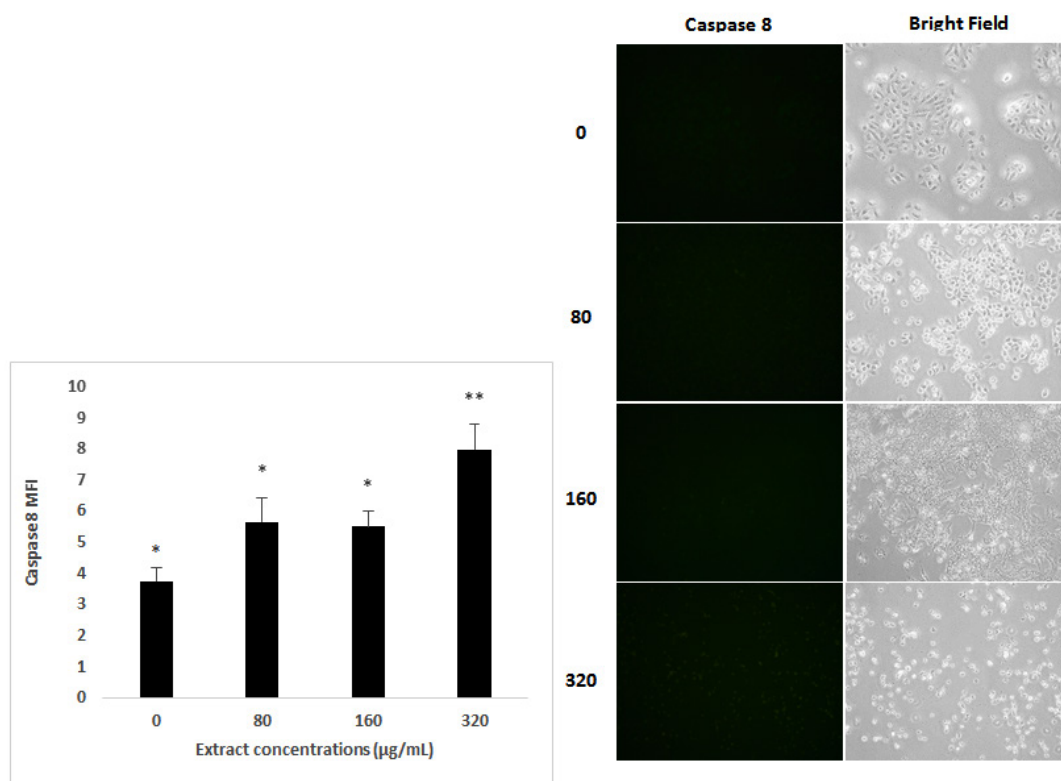
Interestingly, caspase-8 exhibited a more concentration-dependent response, with a significant increase in immunoreactivity observed only at the highest tested concentration. Caspase-8 functions primarily within the extrinsic apoptosis



**Fig. 3.** Caspase-3 immunofluorescence in T47D cells after treatment with *S. elliptica* ethanolic extract. (Left) Quantification of caspase-3 mean fluorescence intensity (MFI) at 0, 80, 160, and 320 µg/mL (mean ± SD, n = 3). One-way ANOVA followed by Duncan's multiple range test showed that caspase-3 immunofluorescence signals were significantly higher in all treated groups (80, 160, and 320 µg/mL) compared with the control ( $p < 0.05$ ), while no significant difference was observed among the treatment groups. Different symbols indicate significant differences among groups. (Right) Representative immunofluorescence images with corresponding bright-field panels. Green fluorescence represents caspase-3 immunoreactivity.

pathway initiated by death receptor signaling.<sup>31</sup> This pattern may indicate that caspase-8-associated signaling becomes more prominent at higher extract concentrations; however, the present data are insufficient to determine whether the extrinsic pathway represents a primary or secondary mechanism of apoptosis. Because caspase-8 can also participate in crosstalk with mitochondrial apoptotic signaling through BID cleavage, further pathway-specific studies are required to clarify its mechanistic contribution. Together, the Annexin V and caspase findings support an association between extract treatment and apoptosis-related responses, although the specific apoptotic pathways involved require further mechanistic investigation.

To better understand the chemical basis underlying the observed biological activity, metabolite profiling of the extract was subsequently performed using LC-HRMS. A total of 39 metabolites were detected, reflecting the chemical complexity of the ethanolic extract. Among these, choline, L-norleucine, and valine were putatively annotated as the most abundant compounds. One of the putatively annotated compounds, N,N-diethyl-m-toluamide (DEET), is not typically regarded as a plant metabolite and has been reported as an environmental contaminant.<sup>32-34</sup> Its detection should therefore be interpreted cautiously, as it may reflect exogenous contamination or analytical background rather than a genuine constituent of *S. elliptica*.



**Fig. 4.** Caspase-8 immunofluorescence in T47D cells after treatment with *S. elliptica* ethanolic extract. (Left) Quantification of caspase-8 mean fluorescence intensity (MFI) at 0, 80, 160, and 320 µg/mL (mean ± SD, n = 3). One-way ANOVA followed by Duncan's multiple range test showed that caspase-8 immunofluorescence signals at 320 µg/mL were significantly higher than those in the control, 80, and 160 µg/mL groups ( $p < 0.05$ ), while no significant difference was observed among the control, 80, and 160 µg/mL groups. Different symbols indicate significant differences among groups. (Right) Representative immunofluorescence images with corresponding bright-field panels. Green fluorescence represents caspase-8 immunoreactivity.

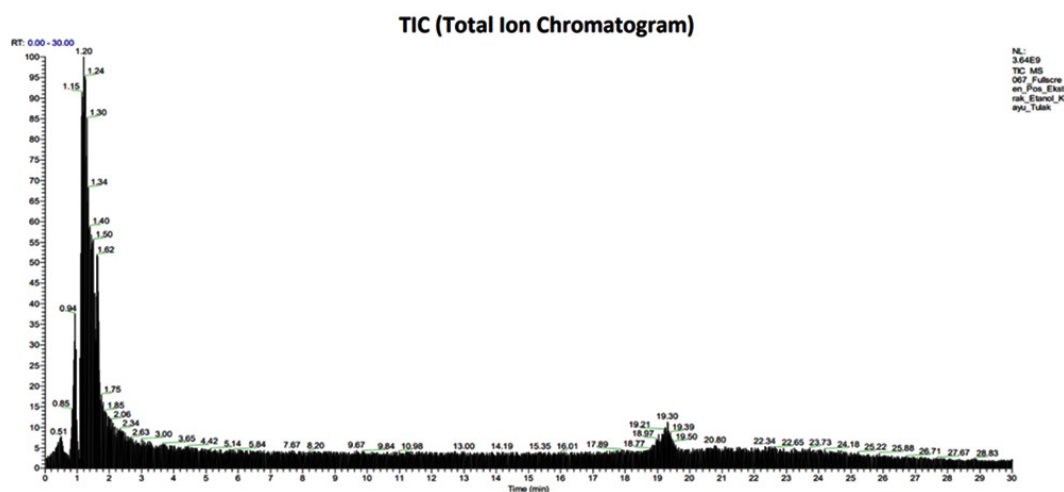
Choline metabolism has been recognized as one of the metabolic hallmarks of cancer. Increased choline kinase- $\alpha$  activity and elevated phosphocholine levels are associated with enhanced cellular proliferation, invasiveness, and therapeutic resistance in several malignancies, including breast cancer.<sup>35, 36</sup> Although the present study did not directly evaluate phospholipid metabolism, the putative annotation of choline indicates that a metabolite associated with this pathway was detected in the extract.

L-norleucine was also detected among the major metabolites. Although L-norleucine itself has

not been extensively investigated as an anticancer compound, structurally related analogs such as 6-diazo-5-oxo-L-norleucine (DON) have been reported to inhibit glutamine-dependent metabolic pathways essential for nucleotide and protein biosynthesis.<sup>37</sup> Because many cancer cells exhibit strong dependence on glutamine metabolism, often referred to as “glutamine addiction”, targeting this pathway has emerged as a promising therapeutic strategy.<sup>38, 39</sup> Likewise, valine, a branched-chain amino acid (BCAA), participates in metabolic reprogramming and the activation of signaling pathways, such as mTOR, that support tumor

**Table 3.** Binding affinity, key interacting residues, and interaction types of venetoclax (reference) and selected *S. elliptica* compounds with Bcl-2 protein

| Ligand       | Binding Affinity (kcal/mol) | Interaction Type(s)                           | Key Residues Involved                                    |
|--------------|-----------------------------|-----------------------------------------------|----------------------------------------------------------|
| Venetoclax   | -7.4                        | Hydrogen bond<br>Hydrophobic                  | ASP103<br>TYR108, ALA149, LEU137, PHE104, ALA100, VAL148 |
| Valine       | -4.3                        | Hydrogen bond<br>Hydrophobic                  | ALA100<br>TYR202, VAL148, PHE198                         |
| Choline      | -3.2                        | Electrostatic<br>Hydrogen bond<br>Hydrophobic | ASP103<br>ALA100<br>TYR202                               |
| L-Norleucine | -4.5                        | Hydrogen bond<br>Hydrophobic                  | ASP103<br>TYR202, VAL148, PHE198                         |



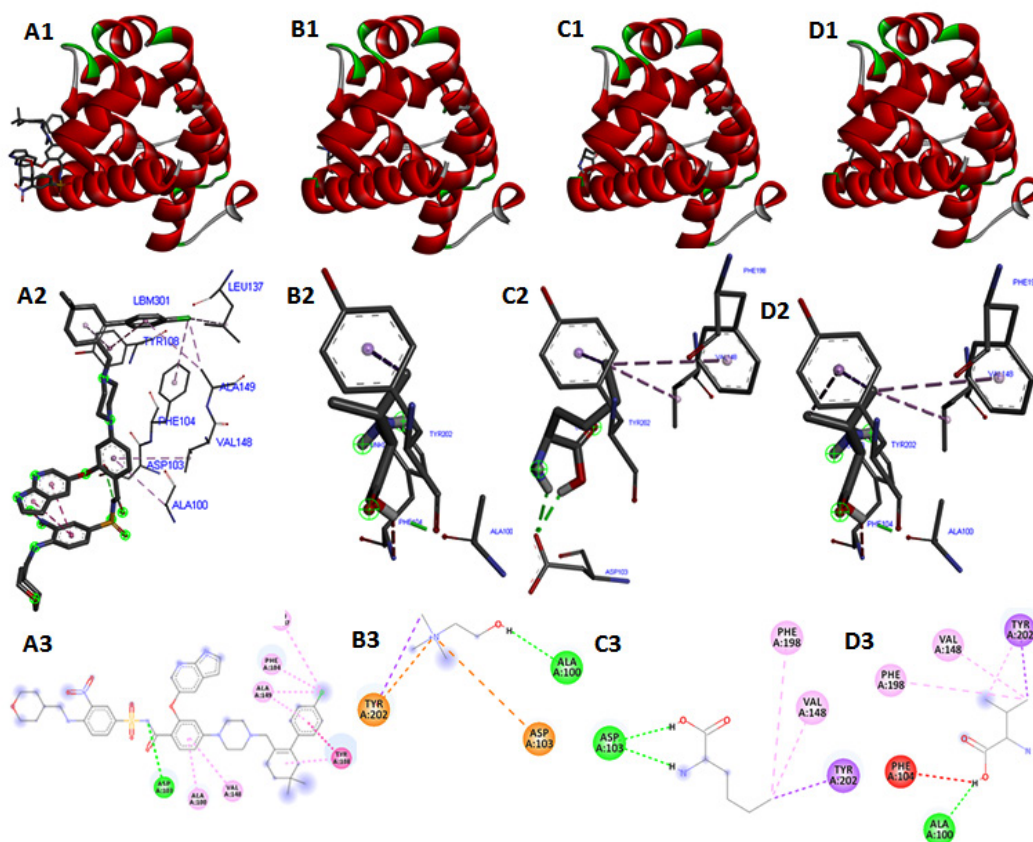
**Fig. 5.** LC/HRMS chromatogram of *S. elliptica* ethanolic extract, showing major peaks corresponding to putatively annotated compounds

growth and survival.<sup>40-42</sup> Nevertheless, it is important to emphasize that LC-HRMS profiling provides putative metabolite annotations but does not establish their biological contribution. Further isolation, structural characterization, and bioactivity-guided fractionation will therefore be required to identify the compounds directly responsible for the observed cytotoxic effects.

To complement the experimental findings, molecular docking analysis was performed to assess whether the metabolites putatively annotated by LC-HRMS could potentially interact with a molecular target involved in apoptosis regulation. Bcl-2 was selected because it is a key regulator of mitochondrial apoptosis that suppresses programmed cell death by binding and sequestering

pro-apoptotic proteins such as BAX and BAK.<sup>43</sup> Consistent with previous structural studies, the reference inhibitor venetoclax exhibited the strongest binding affinity and interacted within the BH3-binding groove of Bcl-2.<sup>21</sup> The three major metabolites evaluated in this study showed lower predicted binding affinities than venetoclax, although several interaction residues—including ASP103, ALA100, TYR202, and VAL148—were shared with the reference ligand. Similar residues have previously been reported in structural studies investigating ligand interactions within the Bcl-2 binding pocket.<sup>44</sup>

Although the selected metabolites exhibited weaker predicted binding affinities than venetoclax, their predicted interactions with



**Fig. 6.** Docking interactions of venetoclax (A1–A3), L-norleucine (B1–B3), valine (C1–C3), and choline (D1–D3) with the Bcl-2 protein. For each ligand, panel 1 shows the 3D complex of the ligand and protein, panel 2 shows the 3D model of the interaction highlighting key residues, and panel 3 presents the 2D model of the interaction illustrating hydrogen bonds, hydrophobic contacts, and electrostatic interactions. Binding affinities are indicated for each ligand, and detailed interactions are summarized in Table 3. Legend: green dashed line = hydrogen bond; purple line = hydrophobic interaction; orange line = electrostatic interaction.

residues within the Bcl-2 binding pocket suggest that Bcl-2 may represent a plausible exploratory target. Because metabolite abundance does not necessarily correspond to biological potency, these findings do not exclude the potential contribution of less abundant constituents to the observed cytotoxic and apoptosis-associated effects. Nevertheless, molecular docking provides computational predictions rather than direct evidence of molecular binding; therefore, further validation using biochemical binding assays or molecular dynamics simulations is required to clarify the relevance of these predicted interactions.<sup>17</sup>

Overall, the present findings suggest that the apoptosis-associated cytotoxic activity of *S. elliptica* ethanolic extract may reflect the combined contribution of multiple bioactive constituents rather than a single dominant metabolite. The integration of biological assays with metabolite profiling and molecular docking provides a framework for future bioactivity-directed investigations, while emphasizing the need for further experimental validation of the underlying molecular mechanisms and predicted interactions.

## CONCLUSIONS

The present study demonstrates that the ethanolic extract of *S. elliptica* exhibits moderate cytotoxic activity against T47D breast cancer cells, with an IC<sub>50</sub> value of 339 µg/mL, accompanied by apoptosis-associated cellular responses as indicated by increased Annexin V-positive cell populations and elevated caspase-3 and caspase-8 immunofluorescence signals. LC-HRMS profiling yielded 39 putatively annotated metabolites, with choline, L-norleucine, and valine among the most abundant. Exploratory molecular docking predicted interactions of these metabolites within the Bcl-2 binding pocket, although these computational findings do not establish direct molecular binding or causality. Overall, the integrated biological, metabolomic, and computational findings support the potential of *S. elliptica* as a source for further bioactivity-guided investigation. Future studies should focus on isolation and structural characterization of the active constituents, evaluation of selectivity toward cancer versus non-cancerous cells, and mechanistic validation

using pathway-specific assays and appropriate *in vivo* models.

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### Conflict of Interest

The authors do not have any conflict of interest.

### Data Availability Statement

Data related to cytotoxicity assays can be accessed through Figshare online repository <https://figshare.com/s/c98cd0185bbdb35a1dce>.

### Ethics Statement

The experiment reported in this paper has received ethical approval from the Health Research Ethics Commission, Faculty of Medicine and Health Sciences, Warmadewa University, Denpasar-Bali under ethics number: 65/Unwar/FKIK/EC-KEPK/X/2023.

### Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

### Clinical Trial Registration

This research does not involve any clinical trials.

### Permission to Reproduce Material from Other Sources

Not applicable.

### Author Contributions

M a d e D h a r m e s t i W i j a y a : Conceptualization, Methodology, Formal Analysis, Data Curation, LC-HRMS Analysis, Writing – Original Draft; Anak Agung Gede Indraningrat: Conceptualization, Resources, Extract Preparation, Investigation, Supervision, Writing – Review & Editing; Wirdatun Nafisah: Methodology, Molecular Docking Analysis, Formal Analysis,

Visualization; Nora Ertanti: Investigation, Cytotoxicity Assay, Apoptosis Analysis, Data Collection.

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