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Anticancer Potency of Methanol Extract from *Terminalia catappa* Leaves Using *In Vitro* and *In Silico* Methods

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Abstract

Terminalia catappa is a plant with potential for application in various antioxidant, anti-inflammatory, antimetastatic and antitumor treatments. Therefore, this study aimed to investigate anticancer properties of methanol extract derived from *T. catappa* leaves, using both *in vitro* and *in silico* methods. The results showed that methanol extract had inhibition of HeLa and DU145 cell lines, with respective IC_{50} values of 352.50 and 954.99 μg/mL. LC-MS/MS analysis identified several active compounds within the extract, including catechin, quercetin, rutin, hirsutrin, loliolide, sesquiterpene, kaempferol, apigenin, cirsiliol, cirsimaritin and demethoxycurcumin, all of which had promising anticancer potential. These compounds performed well in the *in silico* test. According to computational predictions, rutin (−9.1 kcal/mol), catechin (−8.0 kcal/mol) and sesquiterpene (−8.2 kcal/mol) had higher binding affinity values than cisplatin and paclitaxel (−7.5 kcal/mol). Considering these results, the compounds were potentially anticancer agents through the inhibition of the Bcl-2 protein. Finally, *T. catappa* active compounds can be used as alternative candidates as a chemopreventive agent.

Keywords: Antiproliferative, Bcl-2, DU145 cell, HeLa cell, *In silico*, LC-MS/MS, *T. catappa*

Introduction

Cancer is a genetic mutation illness that changes human cell proliferation and disrupts tissue organization [1]. According to the World Health Organization (WHO), the majority of countries in 2015 had cancer as their primary or secondary cause of death before the age of 70 [2]. The number of diagnoses and fatalities reached 18.1 and 9.6 million, respectively, in 2018 [3]. Cervical cancer is one of the kind dangerous cancers affecting women's health. In 2018, there were an estimated 570,000 diagnoses and

311,000 deaths worldwide due to cervical cancer, making it the fourth most prevalent cancer and the fourth leading cause of cancer-related deaths in women [4]. In men, prostate cancer ranks as the second most common cancer diagnosed and the fifth largest cause of cancer-related death globally. In 2018, there were 1,276,106 prostate cancer cases and 358,989 deaths recorded worldwide. This represents 3.8 % of all male cancer-related fatalities and 7.1 % of all diagnosed malignancies [5]. Globally, there is an increase in the number of cases of prostate cancer that are being diagnosed; nevertheless, the incidence of prostate cancer varies over 25 times among different nations [5,6].

Central to understanding cancer pathology is apoptosis, a crucial process governed by the Bcl-2 family of proteins, which includes both proapoptotic and antiapoptotic members. Antiapoptotic proteins (Bcl-2 and B-cell lymphoma-extra-large [Bcl-xl]) protect mitochondrial integrity, prevent mitochondrial membrane potential loss and prevent cell death. These proteins are present in most cancer cells, with proapoptotic proteins being missing or underexpressed [7]. Researchers discovered that Bcl-2 expression in ovarian cancer cells indicates resistance to mTOR inhibitor-induced programmed cell death [8]. Previous immunohistochemical studies revealed increased Bcl-2 protein levels in advanced prostate cancer [9]. The common treatments for cancer include surgery, radiation, chemotherapy, targeted therapy and immunotherapy, while chemotherapy remains one of the most important treatments. Several chemotherapy medications, such as doxorubicin, paclitaxel, cisplatin, 5-fluorouracil, cyclophosphamide, irinotecan and mitomycin C, treat cancer effectively [10]. Advanced or recurrent cervical and prostate cancer appears to respond best to the chemotherapeutic drug cisplatin, a small-molecule platinum complex that was first discovered to suppress bacterial development before being identified as an anticancer agent [11,12]. A common chemotherapeutic strategy for treating recurrent or metastatic cervical cancer involves combination therapy with cisplatin and paclitaxel [11].

The majority of chemotherapy medications used are synthetically generated and have been shown to be hazardous to cancer and normal cells. In contrast, a new study shows that naturally derived phytochemicals from plants have high selective cytotoxicity and little harm towards cancer and normal cells, respectively, offering treatment options for cancer patients [7]. *T. catappa*, a tropical plant renowned for its traditional medicinal applications in treating diverse ailments such as diarrhea, respiratory disorders, hypertension, insomnia and hematuria, is a promising candidate for chemopreventive therapy [13]. A previous study showed that *T. catappa* leaf extract has antioxidant, anti-inflammatory, antimetastatic and antitumor properties [14]. The leaves contain secondary metabolites such as alkaloids, flavonoids, tannins, steroids and phenols [15]. Flavonoids, known to have anticarcinogenic properties can cause apoptosis in the cancer cell, particularly within the Bcl-2 family [7]. A previous study stated that ethanol extract from *T. catappa* leaves could inhibit the growth of SW480 cells [16]. Similarly, Yeh *et al.* [17] verified the suppressive effect of *T. catappa* extract on hepatocellular cancer. The extract significantly inhibited the proliferation, advancement and migration of A549, MCF-7 and MDA-231 cell lines [14].

Previous studies have shown that *T. catappa* has anticancer activity on various cancer cells. However, there is scanty research to determine the anticancer activity of *T. catappa* against cervical cancer (HeLa) cells and prostate cancer (DU145) cells. Therefore, this study aims to investigate the cytotoxic effects of *T. catappa* leaf extract on HeLa and DU145 cell lines, widely recognized models in cytotoxicity, anticancer, antiproliferative and diagnostic investigations [1]. Subsequently, the *in silico* method was adopted to predict the activity of certain proteins interacting with ligands (secondary metabolite compounds). Molecular docking analyses were conducted on secondary metabolite compounds to assess their potential to inhibit the antiapoptotic protein Bcl-2, pertinent to cervical and prostate cancer.

Materials and methods

Materials

The types of equipment used were rotary evaporator vacuum (BUCHI, Switzerland), analytical balances (OHAUS), glassware, LC-MS/MS (Waters, USA), 1.5 mL microtube, 15 mL tube (Nest, USA), 75 mL T-flask, 96 well plates (Nest, USA), biosafety cabinet (Thermo scientific 1300 series a2, USA), centrifuge (Thermo scientific microCL17, USA), CO_2 Incubator (Thermo scientific series 8000DH, USA), microscope (Thermo scientific EVOS XL Core, USA), multimode Reader (Tecan Infinite M200 PRO, Switzerland), a set of Laptop DELL Vostro 14 3000 specific Processor 11th Gen Intel(R) Core (TM) i5-1135G7 @ 2.40GHz 2.42 GHz 12 GB of RAM, Discovery Studio Visualizer and PyMOL, software AutoDock Tools (ADT) 1.5.6, AutoDock Vina, Ligplot, PubChem web server and Protein Data Bank (PDB).

The materials adopted were *T. catappa* obtained from Babakan Madang District, Bogor Regency, West Java, Indonesia, technical methanol, methanol p.a (Merck), HeLa dan DU145 cell culture, distilled water, antibiotic, dimethyl sulfoxide (DMSO) (Sigma Aldrich USA), fetal bovine serum, phosphate buffered saline (PBS), PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, Uppsala, Sweden), RPMI media, trypan blue and trypsin-EDTA. The 3-dimensional structure of the secondary metabolite compounds was downloaded from http://pubchem.ncbi.nlm.nih.gov, while the structure of the Bcl-2 PDB-ID 6QGG target protein was downloaded from http://www.rcsb.org. Finally, the control compounds include cisplatin (EDQM, European Pharmacopoeia, France) and paclitaxel (TCI Europe NV, Belgium).

Extraction

T. catappa leaves from Babakan Madang District, Bogor Regency, West Java, Indonesia were cleaned, divided and dried in the shade at room temperature (28 ± 2 °C). Subsequently, the dried leaves were finely powdered using a dry grinder and stored in an airtight container at −20 °C. A total of 100 g of dried powder was macerated in methanol at a ratio of 1:10 (w/v) for 3 cycles of 24 h each at room temperature, stirred periodically, followed by concentration using a rotary vacuum evaporator at 47 °C for 6 h. Extracts were prepared in triplicate and the yield was calculated [18].

Cell lines and culture

HeLa and DU145 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco), supplemented with 10 % fetal bovine serum (FBS, Gibco) and 50 µL/ 50 mL antibiotic. Cultures were maintained under standard conditions at 37 °C in an incubator containing 5 % CO_2. Cells of a confluent monolayer were harvested by scraping, washed 3 times with 10 mM PBS, pH 7.4 (PBS) and counted with a hemocytometer [19].

Cell viability assay

According to Izdihar *et al.* [20], the PrestoBlue assay was used to perform the cytotoxic bioassay. Several resazurin-based cell types were subjected to rapid and quantitative analysis of their proliferation using live-cell reduction capabilities using the Presto Blue reagent (Thermo Fisher Scientific, Uppsala, Sweden). When cells are living and in good condition, their cytosolic environment is reduced. Reducing absorbance or fluorescence outputs reduces resorufin (purple), which functions as a cell viability indicator by reducing resazurin (blue). The conversion is correlated with the number of metabolically active cells. Additionally, cell lines HeLa and DU145 that had reached 70 % confluency were extracted, counted and diluted using RPMI media for full culture. After that, the cells were divided into 96-well plates, with

170,000 cells per well. After growing overnight, they were treated with several doses of *T. catappa* leaves extract (62.5, 125, 250, 500 and 1,000 µg/mL) in PBS with a co-solvent of 2 % (v/v) DMSO. For a duration of 24 h, the samples were incubated at 37 °C in an incubator with 5 % CO_2 and cisplatin as the positive control. Following incubation, the medium was quickly changed to a 90 µL RPMI medium containing 10 µL of PrestoBlue reagent. After 1 to 2 h of incubation, the plate's color changed from blue to purple, indicating the formation of resorufin. At 570 nm, the absorbance was determined with a multimode reader (Tecan Infinite M200 PRO, Switzerland). Furthermore, the concentration at which 50 % of growth is inhibited is known as the IC_{50} value. The 50 % cytotoxicity (IC_{50}) was shown in a plot showing % cytotoxicity against sample concentrations. Ultimately, every analysis and assay were run in triplicate and averaged.

Identification of active compounds by liquid chromatography-mass spectrometry (LC-MS/MS)

A total of 1.4 mg of *T. catappa* extract was dissolved in 100 mL of methanol. The solution was then filtered with a 0.2 m GHP filter and injected into the UPLC system. LC-MS analysis was performed using a Xevo-ToF-1 system equipped with a C-18 column (particle dimensions: 1.8 µm, 2.1×100 mm^2) and MS with Xevo G2-S resolution QTOF acquisition mode ESI (-) and MSE. The eluent comprised 0.1 % formic acid in distilled water (A) and 0.1 % formic acid in acetonitrile (B), at a running time of 20 min and a temperature of 100 °C. The solvent ratio during elution included 70 % A and 30 % B at 0 - 1 min, 5 % A and 95 % B at 6 - 18 min, as well as a linear gradient to 70 % A and 30 % B at 19 - 20 min. Data processing was conducted using the MassLynx 4.1 program [21].

Collection of 3D ligand and protein

Structure secondary metabolite compounds of *T. catappa* leaves extract were collected in *sdf format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and transformed to *pdbqt format using Marvin sketch. The anti-apoptotic protein inhibitor Bcl-2 was positively controlled by cisplatin CID 5702198 and paclitaxel CID 36314. The 3D structure of the target protein in the form of B-cell lymphoma 2 (Bcl-2) protein ID: 6QGG was downloaded from the Protein Data Bank (PDB) site and prepared using 2 software [22]. Furthermore, the first preparation comprised the usage of Notepad^{++} software to remove impurities such as water content and native ligands. The receptor was saved in .txt format, then the .txt file was prepared using AutoDock Tools-1.5.6 software to attach polar hydrogen atoms and Kolman charges. Finally, the protein structure obtained was saved in *pdbqt format.

Molecular docking and cytotoxic prediction

The docking process commenced with the determination of the grid box to identify the ligand's rotation space relative to the receptor position. The coordinates of the grid box used were x = 1.566, y = −26.511 and z = 87.344, with spacing of 1 Å. Molecular docking simulations were conducted using AutoDock Tools 1.5.6 by modifying the ligand and protein structures in *pdbqt format. All molecular structure and grid box data were stored in 1 folder within a working directory in *pdbqt format. Docking was performed using AutoDock Tools 1.5.6 and AutoDock Vina software, invoked through the Command Prompt. Each simulated ligand was replicated to obtain the best Gibbs free energy (ΔG), with accepted ΔG range criteria ranging from −5 to −10 kcal/mol and smaller than cisplatin and paclitaxel controls. Subsequently, CLC-Pred (Cell Line Cytotoxicity Predictor web server) via https://www.way2drug.com/Cell-line/ was adopted to predict SMILES ligands retrieved from the PubChem database. The final stage comprised visualizing the docking results using Ligplot^{+} 4.3.5 and Biovia Disc Studio 2021 software.

Statistical analysis

Statistical analyses were conducted between treated and non-treated groups using analysis of variance. For all statistical tests, a significance level of $\alpha = 0.05$ was considered.

Results and discussion

Antiproliferative effects of HeLa and DU145 cell lines

The results of extracting *T. catappa* leaves with methanol obtained an extract yield of 5.26 ± 0.10 %. Therefore, the extract was tested for *in vitro* cytotoxic activity against HeLa and DU-145 cell lines. The results are shown in **Table 1**.

Table 1 *In vitro*, cytotoxic activity of *T. catappa* leaves methanol extract in cell lines.

Concentration (µg/mL)	% cell viability	
	HeLa	DU145
0	100.00 ± 0.99	100.00 ± 0.63
62.50	100.00 ± 0.92	100.00 ± 0.63
125.00	99.75 ± 0.24	99.08 ± 0.49
250.00	77.80 ± 8.31	93.94 ± 3.57
500.00	19.72 ± 5.98	73.97 ± 7.64
1000.00	1.30 ± 0.98	42.93 ± 8.37
Cisplatin (control)	11.60 ± 1.85	34.39 ± 6.01

The cytotoxicity of methanol extract against both the cell lines was screened by PrestoBlue assay at different concentrations up to 1,000 µg/mL for 24 h. Results exhibit a dose-dependent inhibition curve at 50 % with the inference of IC_{50} value of 352.50 µg/mL in HeLa cells and 954.99 µg/mL in DU145 (**Figure 1(a)**) compared to positive control (Cisplatin) with concentrations of 19 and 24 µM for HeLa and DU145 cell lines, respectively considering untreated negative controls (HeLa-**Figure 1(b)**, DU145-**Figure 1(e)**). The reduction in the number of cells was discovered to restrict cell proliferation and suggest disruption of cell structure. Morphological changes due to methanol extract-treated studied cell lines (HeLa and DU145) were observed under a microscope (EVOS™ XL Core, Thermo Fisher Scientific, Carlsbad, CA, USA). Typically, HeLa and DU145 cell lines had epithelial and polygonal shapes. Treatment with *T. catappa* leaf extract with a dose of 500 µg/mL (**Figures 1(c) - 1(f)**) and cisplatin (**Figures 1(d)** and **1(g)**) changed the appearance of the normal cells, which were found to be irregular, aggregated, and spherical, indicative of restricted damaged cells and spreading patterns. The results showed that *T. catappa* leaf extract considerably reduced cancer cell viability and has potential anticancer effects, particularly against cervical cancer. In a previous study, the ethanol extract of endophytic fungi from *T. catappa* stem bark had an IC_{50} value of 33.35 µg/mL against HeLa cell lines [23], and the ethanol extract from leaves yielded 46.79 µg against SW480 cells [16].

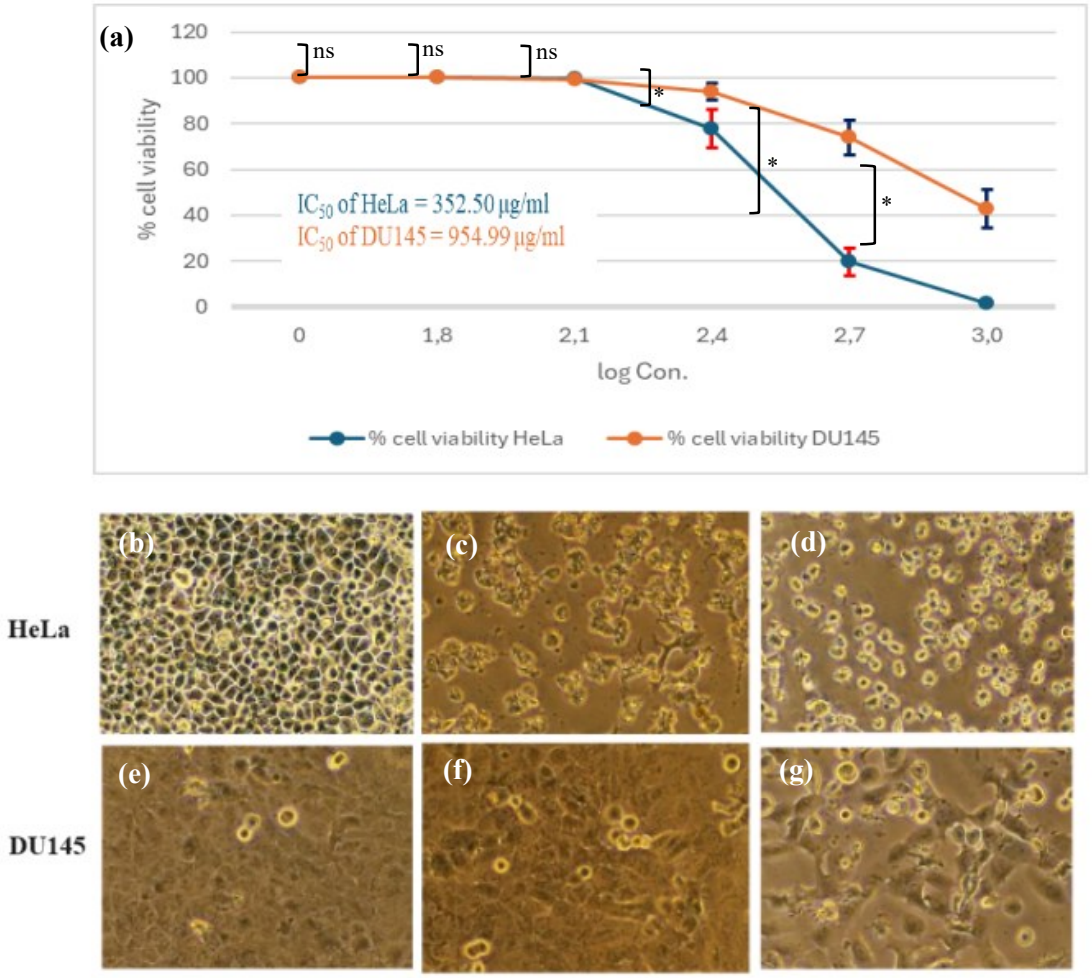

Figure 1 Cytotoxicity measured by PrestoBlue assay on HeLa and DU145 cell lines. (a) IC50 value of leaf extract treated cell lines. Normal untreated negative control: (b) HeLa cell lines and (e) DU145 cell lines. Morphological changes due to 500 µg/mL of leaf extract on: (c) HeLa cell lines and (f) DU145 cell lines. Morphological changes due to 19 and 24 µM of cisplatin on: (d) HeLa and (g) DU145 cell lines, respectively. Notes: *p*-values are shown as follows: ns, non-significant, *$p < 0.05$ is considered as significant.

Results of identification of secondary metabolite compounds using LC-MS/MS

Secondary metabolite compounds of *T. catappa* leaves methanol extract were identified using LC-MS/MS to obtain data from chromatograms and mass spectra. The chromatogram of this extract is shown in **Figure 2**. Based on LC-MS/MS identification results, there were 53 compounds, 11 comprising secondary metabolites with potential anticancer properties. These compounds are presented in **Table 2**.

Table 2 Alleged compounds resulting from LC-MS/MS of methanol extract of *T. catappa* leaves.

No	RT (Min.)	% Area	Parent ion $[M + H]^+$	Product ion (m/z)	Compound name	Class
1	3.805	1.02	291.0867 $C_{15}H_{14}O_6$	163.04	Catechin	Flavonoid
2	4.311	0.91	303.0505 $C_{15}H_{10}O_7$	163 287	Quercetin	Flavonoid
3	4.621	1.90	611.1612 $C_{27}H_{30}O_{16}$	147 303	Rutin	Flavonoid
4	4.663	1.90	465.1046 $C_{21}H_{20}O_{12}$	161 257.04 303.05	Hirsutrin	Flavonoid
5	5.914	0.15	197.1179 $C_{11}H_{16}O_3$	137.06 161.096 179.1072	Loliolide	Benzofuran
6	7.383	2.90	247.1334 $C_{15}H_{18}O_3$	133.10 221.117 229.122	Sesquiterpene	Terpenoid
7	7.433	3.16	287.0556 $C_{15}H_{10}O_6$	161.0 225.0	Kaempferol	Flavonoid
8	7.940	3.16	271.0606 $C_{15}H_{10}O_5$	229 211	Apigenin	Flavonoid
9	8.115	0.86	331.0818 $C_{17}H_{14}O_7$	151.03 313.07 331.08	Cirsiliol	Flavonoid
10	9.430	1.25	315.0869 $C_{17}H_{14}O_6$	123.0 271.0	Cirsimaritin	Flavonoid
11	11.053	0.42	339.1234 $C_{20}H_{18}O_5$	115 219	Demethoxycurcumin	Curcumin

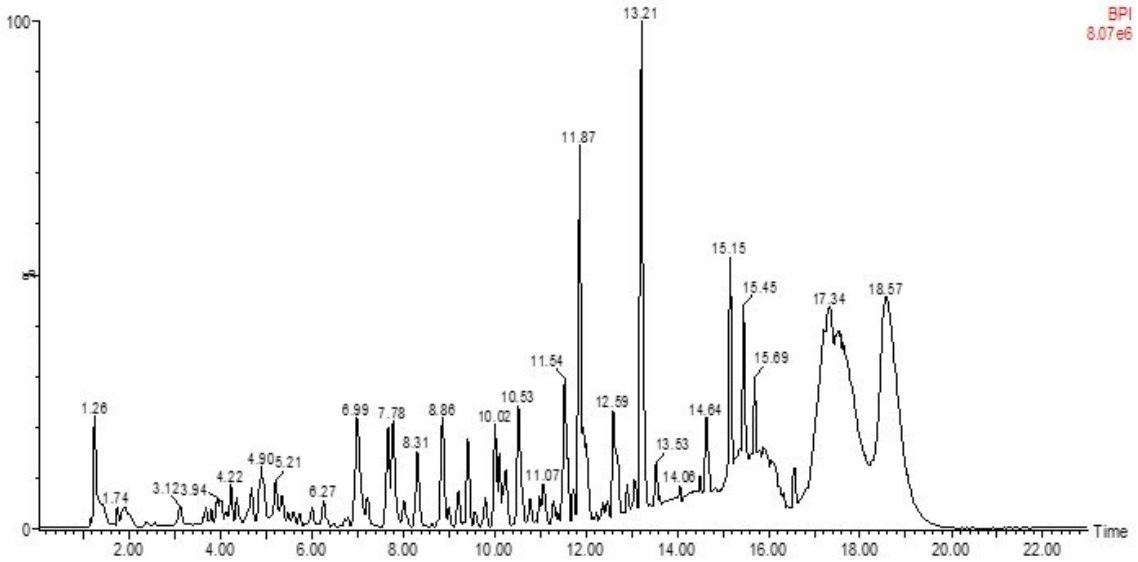

Figure 2 Chromatogram of methanol extract of *T. catappa* leaves using LC-MS/MS.

The flavonoid group dominates the secondary metabolites in *T. catappa* leaves. The compounds are polyphenolic and have strong antioxidant properties due to the several hydroxyl groups within the molecules. Extensive *in vitro* and *in vivo* studies have shown the potent antioxidant activity of these compounds, contributing to a reduction in cancer risk [24]. By scavenging free radicals and alleviating oxidative stress, flavonoids play a crucial role in combating various diseases. Furthermore, the compounds exerted diverse anticancer effects, including cell cycle arrest, induction of apoptosis and autophagy, as well as inhibition of cancer cell proliferation and invasion [25].

The retention time of 3.805 was shown in the spectrum, as detailed in **Figure 3**. A compound known as catechin ($C_{15}H_{14}O_6$) (1), was suspected to have anticancer activity, with a molecular weight of $[M+H]^+$ 291.0867 m/z. Catechins are a class of flavonoid compounds from the flavanol group. A study conducted *in vitro* shows that catechins isolated from gambier plants have cytotoxic activity, thereby inhibiting HeLa cell migration and inducing apoptosis in the culture cell [26]. A recent *in silico* investigation by Fadlan *et al.* [27] reported the anticancer function of the compounds by inhibiting the protein death-associated protein kinase 1 (DAPK1).

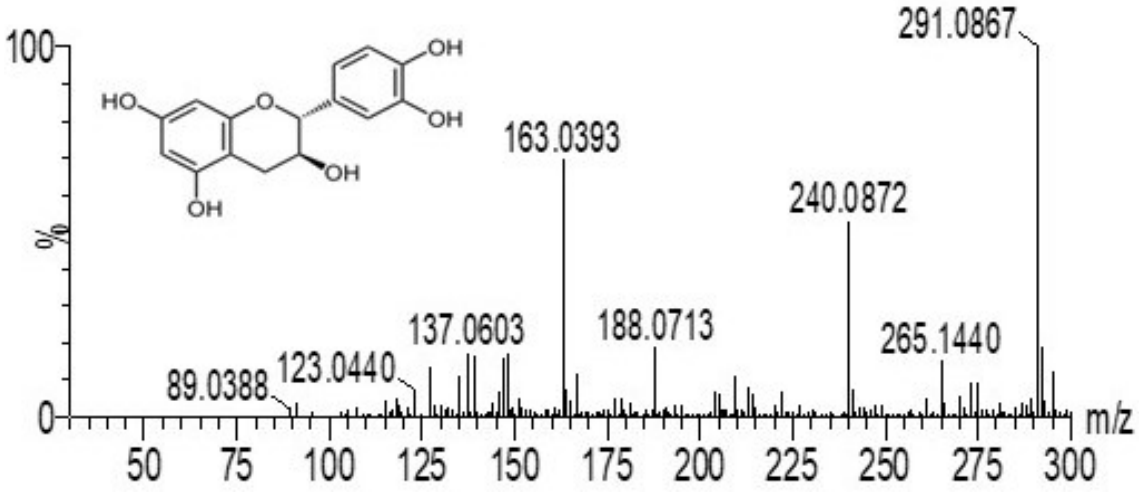

Figure 3 The LC-MS/MS spectrum of methanol extract at a retention time of 3.805.

The retention time of 4.621 is shown in the spectrum (**Figure 4**). A compound known as rutin ($C_{27}H_{30}O_{16}$) (3), a class of flavonoid compounds from the flavonol group, was suspected to have anticancer capabilities, with a molecular weight of $[M + H]^+$ 611.1612 m/z. Previous studies have stated its potential to inhibit cell attachment and migration, thereby reducing proliferation and decreasing ROS production in the lung (A549) and colon cancer cell lines (HT29 and Caco-2) [28].

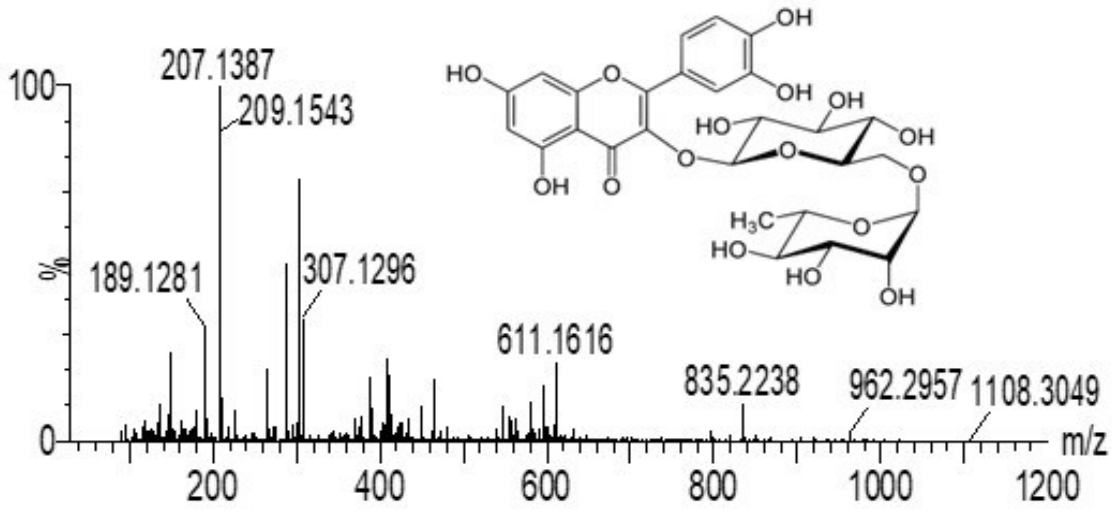

Figure 4 The LC-MS/MS spectrum of methanol extract at a retention time of 4.621.

In addition to flavonoid compounds, methanol extract of *T. catappa* leaves also contains terpenoid compounds, namely sesquiterpene ($C_{15}H_{18}O_3$) (6). This compound was identified at a retention time of 7.383 (**Figure 5**) and had a molecular weight of $[M + H]^+$ 247.1334 m/z. Sesquiterpene compounds isolated from *Melampodium leucanthum* leaves extract were known to be cytotoxic against PC-3 and DU 145 prostate cancer cells, as well as HeLa cervical cancer cells, with IC_{50} values ranging from 0.18 to 9 μM [29].

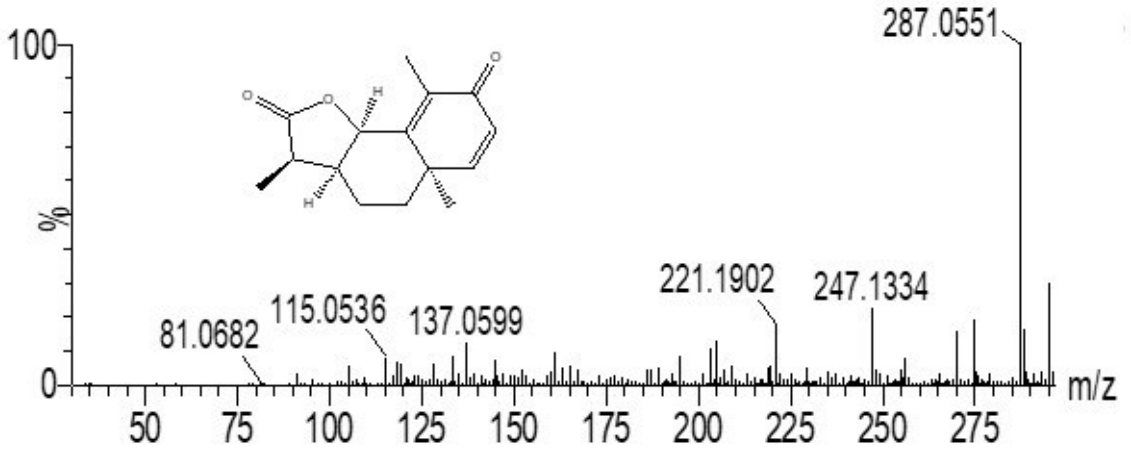

Figure 5 The LC-MS/MS spectrum of methanol extract at a retention time of 7.383.

Molecular docking and visualization

The potential of the secondary metabolite compounds of *T. catappa* leaves methanol extract as anticancer was predicted using the docking method. This method enabled the identification of new drug candidates by predicting and visualizing the interaction between target proteins and testing ligands at the molecular level. Molecular docking was conducted using Autodock Vina software to analyze chemical bonds and determine binding affinity values or Gibbs free energy (ΔG) between the target protein and test ligand. The research showed that 3 compounds produced the lowest ΔG and had inhibitory activity against the Bcl-2 protein: Rutin, sesquiterpenes and catechins. These compounds had Gibbs free energy values of

−9.0, −8.2 and −8.0 kcal/mol, respectively. In contrast, the comparison ligands, namely paclitaxel and cisplatin, had the same Gibbs free energy value of −7.5 kcal/mol, as presented in **Figure 6**.

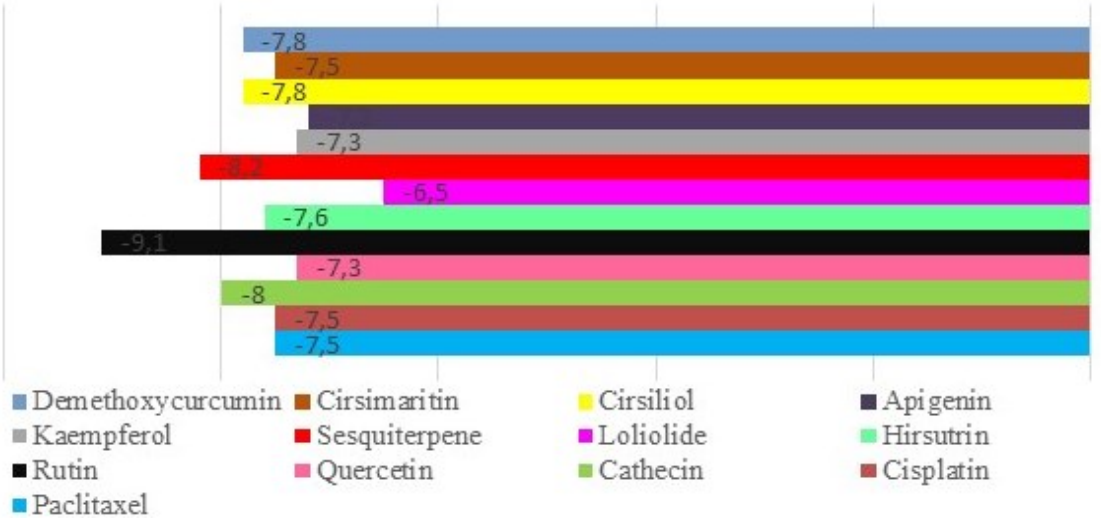

Figure 6 The binding affinity value resulting from the docking of secondary metabolite compounds from *T. catappa* leaves with receptors Bcl-2.

Based on analysis, numerous bonds, including hydrogen, van der Waals and hydrophobic, are formed between ligand and receptor. In protein-ligand interactions, hydrogen bonds and hydrophobic interactions affected the stability of the binding between the ligand and its target. The 2 molecules produced strong bonds when enough combination of hydrogen and hydrophobic interactions was established [30]. The results of the binding between the ligand and Bcl-2 receptor are shown in **Table 3**.

Table 3 The amino acid residue ligand-receptor Bcl-2.

No.	Ligand Name	Van der Waals interanctions	Hydrophobic bond			Hydrogen bond	
			Pi bond	Alkyl bond	Carbon Hydrogen bond	Residue	Distance (Å)
1	Paclitaxel	Asn A:143 Asp A:111 Glu A:152 Val A:156 Phe A:153 Ala A:149 Glu A:136 Leu A:137 Arg A:139 Phe A:112	Asp A:140 Tyr A:108	Phe A:104 Met A:115	-	Arg A:146 (N-O)	2.87
2	Cisplatin	Gln A:118 Phe A:153 Val A:133 Asp A: 111 Phe A:104	Glu A:114 Tyr A:108	Met A:115 Leu A:137 Ala A:149	-	Glu A:136 (O-O) Arg A:139 (N-O) Arg A:139 (N-O) Asp A:140 (O-O)	2.82 2.89 3.15 3.19
3	Cathecin	Phe A:112	Phe A:108	Ala A:149	-	Asp A:111	3.82

No.	Ligand Name	Van der Waals interanctions	Hydrophobic bond			Hydrogen bond	
			Pi bond	Alkyl bond	Carbon Hydrogen bond	Residue	Distance (Å)
		Glu A:152	Tyr A:108	Met A:115			
		Phe A:153					
		Leu A:137					
		Gly A:145					
		Arg A:146					
4	Quercetin	Phe A:112	Leu A:137	Leu A:137	-	Ala A:149 (O-O)	2.86
		Phe A:104	Glu A:152	Phe A:153		Asp A:111 (O-O)	2.70
				Met A:115		Glu A:136 (O-O)	2.93
						Arg A:146 (N-O)	3.02
5	Rutin	Asn A:143	-	Met A:115	Asp A:140	Arg A:139 (N-O)	3.06
		Gly A:145		Ala A:149			
		Phe A:104		Arg A:146			
		Phe A:112					
		Phe A:153					
		Asp A:111					
		Leu A:137					
		Arg A:146					
		Glu A:136					
6	Hirsutrin	Glu A:114	Leu A:137	Ala A:149	Phe A:112	Arg A:146 (N-O)	3.22
		Met A:115	Phe A:104			Asp A:111 (O-O)	3.24
		Val A:133				Glu A:136 (O-O)	3.21
		Phe A:153				Ala A:149 (O-O)	2.70
		Glu A:152					
		Ser A:105					
7	Loliolide	Ala A:100	Tyr A:202	Phe A:104	-	Arg A:107 (N-O)	3.00
		Phe A:198					
		Trp A:144					
		Val A:148					
		Gly A:145					
		Tyr A:108					
8	Sesquiterpene	Arg A:107	Tyr A:202	Val A:148	-	Tyr A:108 (O-O)	3.08
		Phe A:104		Ala A:100			
		Gly A:145		Phe A:198			
9	Kaempferol	Leu A:137	Gly A:145	Ala A:149	-	Asn A:143 (N-O)	3.27
		Tyr A:108	Phe A:104	Val A:148		Ala A:100 (O-O)	2.93
		Arg A:107		Arg A:146			
		Asp A:103					
10	Apigenin	Leu A:137	Gly A:145	Ala A:149	-	Ala A:100 (O-O)	2.93
		Asn A:143	Phe A:104	Val A:148		Asn A:143 (N-O)	3.27
		Tyr A:108		Arg A:146			
		Arg A:107					
		Asp A:103					
		Ala A:100					
11	Cirsiliol	Tyr A:108	Gly A:145	Arg A:146	-	Arg A:146 (N-O)	2.96
		Arg A:107	Phe A:104	Ala A:149		Asn A:143 (N-O)	3.19
		Asp A:103		Leu A:137			

No.	Ligand Name	Van der Waals interanctions	Hydrophobic bond			Hydrogen bond	
			Pi bond	Alkyl bond	Carbon Hydrogen bond	Residue	Distance (Å)
				Val A:148			
12	Cirsimaritin	Tyr A;108	Gly A:145	Ala A:149	-	Arg A:146 (N-O)	2.89
		Arg A:107	Phe A:104	Leu A:137		Asn A:143 (N-O)	3.32
		Asp A:103		Val A:148			
13	Demethoxycurcumin	Asp A:103	Phe A:104	Ala A:149	-	Ala A:100	4.49
		Arg A:107		Leu A:137			
		Tyr A:108		Met A:115			
		Phe A:153		Val A:148			
		Phe A:112					
		Asp A:111					
		Arg A:146					
		Gly A:145					

Based on **Table 3**, the test ligands that have similar interactions with the comparison ligand paclitaxel include rutin and catechin, respectively, the similarity of interactions based on the number of amino acid residues is 80 and 60 %. The presence of similar amino acid residues suggested that the test compound has the same potential biological activity as the comparison ligand as an anticancer agent. An amino acid residue that interacts with rutin and catechin, as well as the comparison ligand for paclitaxel, was Arg146. Arginine (Arg) residue is known to directly activate cell proliferation and apoptosis through engagement in various signaling pathways [31].

The cytotoxic action of compounds (ligands) against cancer cell lines was predicted using the website CLC-Pred (Cell Line Cytotoxicity Predictor). The website was used to forecast cytotoxicity based on the cytotoxic association of cancer cell line structures created in the PASS training sets. The results showed an accuracy of 96 % based on *in vivo* studies. This prediction can facilitate the virtual screening of potential anticancer agents *in silico*. The outcomes of CLC-Pred are represented by Pa (Possibility to be active) and Pi (Possibility to be inactive) values [32].

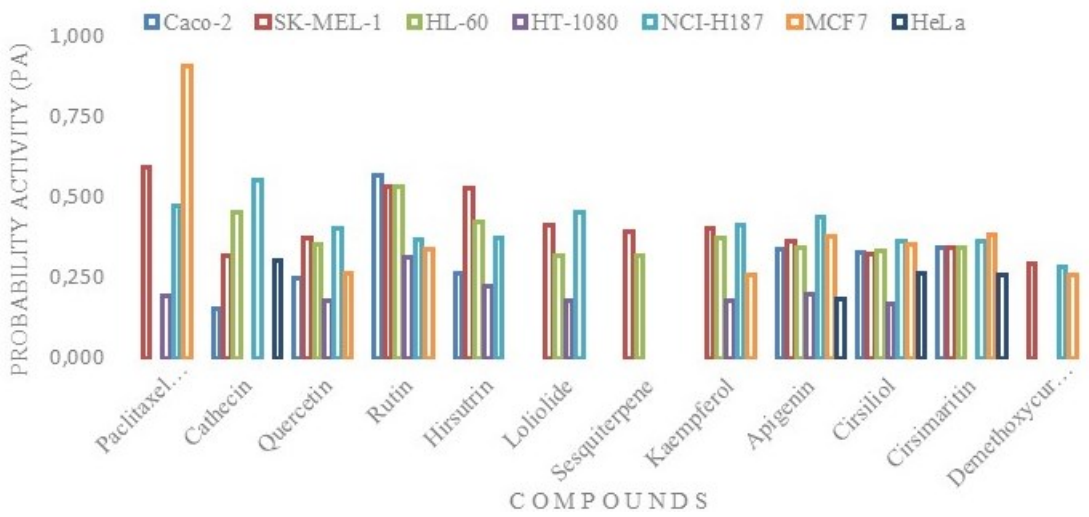

Figure 7 Cytotoxic activity of compounds (ligands) against several cancer cell lines.

According to the CLC predictions shown in **Figure 7**, the secondary metabolite compounds from *T. catappa* have cytotoxic potential in various cancer cell lines. Some of the cancer cell lines include Caco-2 (Colon adenocarcinoma in the colon), SK-MEL-1 (Metastatic melanoma in the skin), HL-60 (Promyeloblast leukemia in the hematopoietic and lymphoid tissue), HT-1080 (Fibrosarcoma in the soft tissue), NCI-H187 (Small cell lung carcinoma in the lung), MCF7 (Breast carcinoma in the breast) and HeLa (Cervical adenocarcinoma in the Cervix). However, the cytotoxic activity of these compounds against DU145 cells has not been found in CLC-Prediction. Compounds with Pa > 0.5 have a high potential for cytotoxic activity, with laboratory tests often confirming similar results. Those with 0.3 < Pa < 0.5 were considered to have a moderate potential for cytotoxic activity, while Pa < 0.3 showed a low potential when tested on a laboratory scale [32]. Compounds with high potential as anticancer were rutin, catechin and hirsutrin. The docking results showed that these 3 compounds have a lower Gibbs free energy than the comparison ligands such as paclitaxel and cisplatin. Visualization of the docking results between rutin and Bcl-2 receptor, alongside the control ligand, is shown in **Figure 8**.

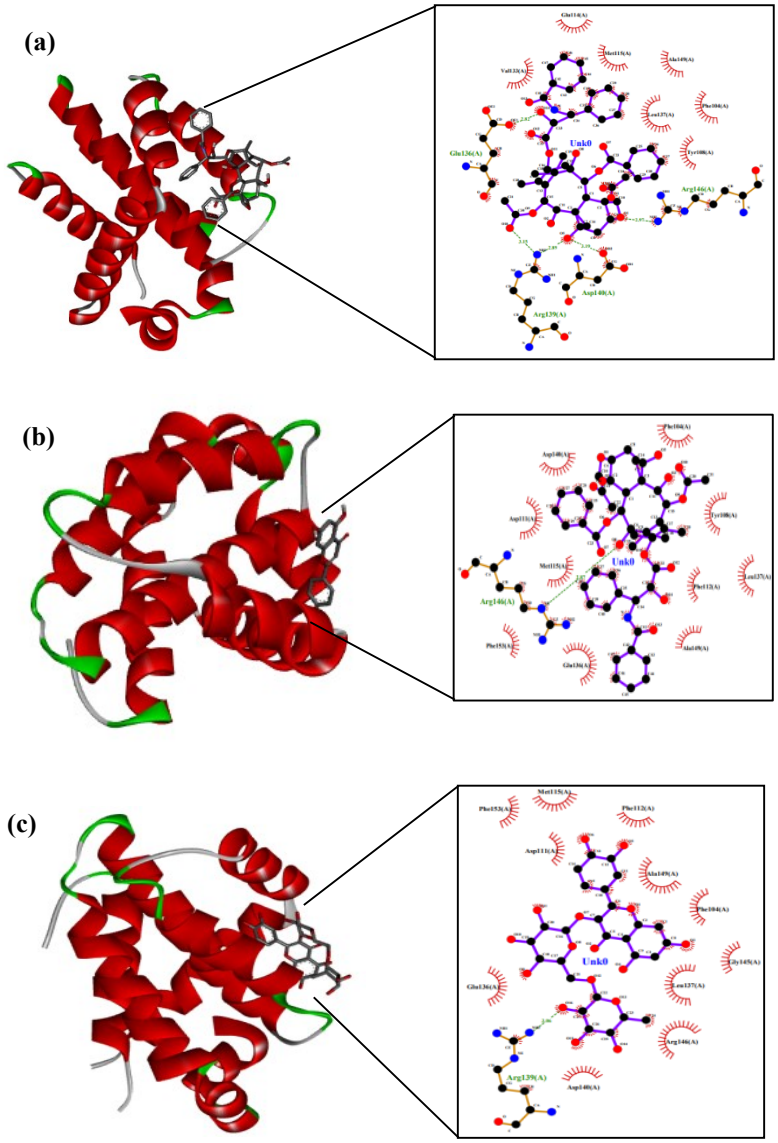

Figure 8 Visualization of the docking results between ligands and Bcl-2 receptor: (a) Cisplatin-Bcl-2 receptor, (b) paclitaxel- Bcl-2 receptor and (c) rutin-Bcl-2 receptor.

Conclusions

In conclusion, methanol extract from *T. catappa* leaves was tested *in vitro* for its antiproliferative and cytotoxic effects on HeLa and DU145 cell lines. The percentage of cellular viability decreased with increasing extract concentration, showing a dose-dependent effect. The IC_{50} values for methanol extract from *T. catappa* leaves against HeLa and DU145 cell lines were determined to be 352.50 and 954.99 µg/mL, respectively. Furthermore, LC-MS/MS analysis identified several active compounds within the extract with potential anticancer properties. These compounds include catechin, quercetin, rutin, hirsutrin, loliolide, sesquiterpene, kaempferol, apigenin, cirsiliol, cirsimaritin and demethoxycurcumin. *In silico* data analysis showed that rutin, catechin and sesquiterpene, had higher bond affinity values than the control compounds (paclitaxel and cisplatin). Based on the results of CLC-Pred, compounds that have the potential to be anticancer agents are rutin, catechin and hirsutrin. However, further studies are required by testing the *in vitro* anticancer activity of the compounds contained in the methanol extract of *T. catappa* leaves against both types of cancer cells (HeLa and DU145) to find out which compounds have the most potential as anticancer agents, especially for cervical and prostate cancer.

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