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## Research Article

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### Anti-Cancer Assessment of a Ramie (*Boehmeria nivea* L. Gaud.) Leaf Extract Using Mcf-7 Cell Line and a Yeast-Based Bioassay

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## ABSTRACT

**Introduction:** One strategy for molecular cancer therapy is to know the key mechanism of cytotoxic compounds that can kill cancer cells. Ramie (*Boehmeria nivea* L. Gaud.) leaves contain active compounds that have important effects on cancer chemoprevention. **Objective:** To obtain the active fraction of a Ramie leaf extract in inhibiting the proliferation of MCF-7 breast cancer cell lines and to determine the mechanism of apoptosis induction using MCF-7 and *Saccharomyces cerevisiae* strains 1140, 1353, and 1138. **Method:** Fractions were prepared using n-hexane, dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), ethyl acetate, and n-butanol as solvents. All fractions were tested qualitatively through phytochemical. The MTT-based cytotoxicity assay used MCF-7 (in vitro) to obtain the  $\text{IC}_{50}$  value, whereas the model system that targets the enzymatic (topoisomerase) used a yeast-based bioassay to obtain the  $\text{IC}_{12}$  value. Apoptotic induction of the active fraction in MCF-7 was performed using flow cytometry and qPCR ( $2^{-\Delta\Delta\text{Ct}}$  method). **Results:** The phytochemical analysis indicated that the extract fraction consisted of alkaloids and steroids. The smallest  $\text{IC}_{50}$  value was obtained from the  $\text{CH}_2\text{Cl}_2$  fraction as 3.79 g/mL, potentially act as an anticancer. A higher percentage of apoptosis than that of necrotizing cells and live cells was observed through flow cytometry. The  $\text{CH}_2\text{Cl}_2$  fraction with an  $\text{IC}_{12}$  value < 8000 in strains 1140, 1353, and 1138 consistently showed the mechanism of apoptosis induction as topoisomerase I and II inhibitors. Also, another mechanism could be through the intrinsic pathway, indicated by the highest expression level in p53. **Conclusions:** The  $\text{CH}_2\text{Cl}_2$  fraction of Ramie leaves can inhibits the proliferation of MCF-7 cells in the active and strong categories. The  $\text{CH}_2\text{Cl}_2$  fraction induces apoptosis by increasing p53 gene expression and inhibiting topoisomerase I and II. Thus, it showed potential as an anticancer drug candidate.

**Keywords:** apoptosis, Ramie leaf extract, fraction, MCF-7, mechanism-based yeast bioassay, q-PCR

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## Background

Cancer arises from the transformation of normal cells into malignant tumor cells. This disease is the leading cause of death worldwide, with up to 9.6 million deaths in 2018. In Indonesia, the prevalence of cancer has increased in the last 5 years. The Basic Health Research of 2018 stated that the prevalence of cancer in Indonesia reached 1.79 per 1,000 people. The most common cancer is breast cancer, with 2.09 million cases [1]. Breast cancer is the highest case in women at 42.1 per 100,000, with an average death rate of 17 per 100,000 [2]. Breast cancer is formed from breast cells that grow uncontrollably [3].

Despite being a serious disease, breast cancer can still be treated depending on the type and stage of cancer. Several tools are used for the diagnostic and evaluation processes, such as mammograms, computerized tomography scans, magnetic resonance imaging, positron emission tomography scans, blood tests, and tumor receptor tests (ER, PR, and HER-2/neu). The results obtained from the evaluation determine the treatment suitable for the patients, such as surgery, lumpectomy, mastectomy, radiation, chemotherapy, hormone therapy, and systemic treatment [4]. These types of treatment often cause trauma and side effects that bother the patient. One way of treating cancer is to utilize compounds contained in natural ingredients. Research and discovery of new anti-cancer drugs are mostly based on the modification of natural products with bioactive compounds from medicinal plants [5]. One of the plants that can be developed as an anticancer drug is Ramie (*Boehmeria nivea* L. Gaud.). The development of Ramie plants as a producer of the textile fiber industry left more than 40% of leaf biomass, which has not been utilized optimally [6]. Various parts of the Ramie plant, such as leaves and roots, are reported to have potential as antioxidants and anti-inflammatory and antifungal agents [7,8].

Apoptosis induction by cytotoxic drugs in cancer cells is a strategy of activating cell death pathways [9]. The inhibition of cancer cell proliferation can also be done by hindering the topoisomerase enzyme. Many antineoplastic drugs target topoisomerase enzymes, such as

camptothecin (topoisomerase I) [10], doxorubicin, daunorubicin, idarubicin, mitoxantrone, etoposide, and teniposide (topoisomerase II) [11]. The mechanism of apoptosis induction of the Ramie leaf ethanolic extract and its anti-cancer activity using a yeast bioassay are not well known. By knowing the active fraction as an anticancer candidate, its chemical properties and mechanisms can be predicted for medical applications. Based on this description, the objectives of this study were to obtain the active fraction of a Ramie leaf extract in inhibiting the proliferation of MCF-7 breast cancer cell lines and to determine the mechanism of apoptosis induction using MCF-7 and *Saccharomyces cerevisiae* strains 1140, 1353, and 1138.

## Methods

### *Ramie leaf extract fractionation*

The Ramie leaves were obtained from the Lembang clone, originating from the hemp plantation in the Wonosobo area, Central Java, Indonesia. The extraction and fractionation preparations were based on the Otsuka (2006) method in Parsaee et al. [12]. A concentrated Ramie leaf ethanol extract was first dissolved in distilled water and methanol (9:1). The extract solution was fractionated successively using water and other solvents, namely n-hexane, dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), ethyl acetate (EtOAc), and n-butanol (n-BuOH). All fractions were evaporated with a rotary evaporator, and the products were then stored at 4 °C until they were used.

### *Phytochemical test*

The ethanol extract of Ramie leaves and its fractions were tested with qualitative phytochemicals. In the flavonoid test, two to three drops of 2N HCl and sufficient Mg powder were added to the drop plate. The samples contain flavonoids if there is a color change of the solution to orange, red, or brown [13].

Samples were then tested with Dragendorff, Mayer, and Wagner reagents. The samples are alkaloid positive if they have an orange to brown precipitate in the Dragendorff reagent, a red one in the Mayer reagent, and a brown one in the Wagner reagent [14]; steroids and triterpenoids assay on the extracted

samples [14,15]. The saponin test was carried out based on the method of Bintoro et al. [16].

#### **Cytotoxicity test with an MTT assay**

MCF-7 breast cancer cell line was obtained from the Laboratory of Cell and Tissue Culture, the Teaching Hospital, Faculty of Medicine, Padjadjaran University. The cells were cultured in an RPMI 1640 medium supplemented with 10% fetal bovine serum and a 1% antibiotic solution under a 5% CO<sub>2</sub> atmosphere at 37 °C. To evaluate the effect of the Ramie leaf ethanol extract and its fractions on cell viability/proliferation, cells were cultured on a 96-multiwell culture plates with a density of 0.8 × 10<sup>4</sup>–1 × 10<sup>4</sup> cells/well. After 24 hours, the medium was replaced with a fresh medium containing samples of a Ramie leaf fraction at different concentrations (0.1, 1, 10, 100, and 1000 g/ml) and control (0 g/ml).

An in vitro cytotoxicity test was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. After 48 hours of incubation with the extract, MTT was added at a final concentration of 0.5 mg/ml, and the mixture was incubated for 2 hours. The culture medium was discarded, and the absorbance value was then read at a wavelength of 550 nm using an ELISA plate reader.

Observation results were noted as a percentage of cell viability/proliferation relative to the control, which was carried out in a triplicate. The percentage of cell viability is calculated using the following formula:

$$\% \text{ Cell viability} = \left( 1 - \frac{\text{Average absorbance of duplicate sample wells}}{\text{Average absorbance of control wells}} \right) \times 100 \text{ \%}.$$

The dose-response curves, to calculate the IC<sub>50</sub> values, were plotted using Probit Analysis. The morphological changes, including cell shrinkage, membrane blabbing, and rounded and separated cells, caused by the extracts were observed using an inverted microscope with 200× magnification.

#### **Flow cytometry**

Cells were cultured in six-well plates with an RPMI 1640 culture medium and treated

with the active fractions at concentrations of 1/2 IC<sub>50</sub>, IC<sub>50</sub>, and 3/2 IC<sub>50</sub>. Each well contained 1 × 10<sup>7</sup> cells in a 1.5 mL culture medium, incubated in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37 °C) for 48 h. Sample preparation was carried out by administering 0.25% Trypsin-EDTA to the cultured cells. Cells were resuspended in a complete culture medium and transferred to 15 mL polypropylene tubes and centrifuged at 2,500 rpm for 5 minutes at 10 °C. The cell pellet was then resuspended with 1 mL PBS.

The sample (50 µL) was added into a microtube containing 500 µL PBS and then centrifuged at 2,500 rpm for 5 minutes at 10 °C to obtain cell pellets. Cells were resuspended in 50 µL of an Annexin V - PI staining solution in PBS: 1 µL of an Annexin V stock solution (2.5 mg/mL), 1 µL of a PI (propidium iodide) stock solution (2.5 mg/mL), and 50 µL of PBS 1X. After being incubated for 40 minutes at 37 °C, 3 mL of PBS was added and centrifuged at 1,500 rpm for 5 minutes. The pellet was resuspended at 500 µL PBS for analysis on flow cytometry. The flow cytometer data were analyzed using Accuri C6 software.

#### **Expression of p53, Bcl-2, Bax, and caspase-8 using the qPCR method**

Cells were cultured in six-well plates with an RPMI 1640 culture medium, and the active fractions were added with concentrations of 0 g/mL as control, 1/2 IC<sub>50</sub>, IC<sub>50</sub>, and 3/2 IC<sub>50</sub>. The cultures were incubated in an incubator (5% CO<sub>2</sub>, 37 °C) for 48 hours. RNA isolation was performed using the ZYMO RESEARCH kit. The cDNA was synthesized from 1.5 µg of total RNA by incubating it for 1 hour at 42 °C with M-MLV reverse transcriptase [Frementas, Lithuania] and oligo primers [dt] according to the instructions in the kit. Then, 2.5 µL of the reaction mixture was subjected to Polymerase Chain Reaction (PCR) to amplify the sequences of p53, Bcl-2, Bax, Caspase-8, and β-Actin using specific primers (Table 1).

**Table 1. Specific Primers for p53, Bcl-2, Bax, Caspase-3, and β-Actin Genes**

<i>p53</i>	Forward :	5'- TTCCCTGGATTGGCCAGACT-3'
	Reverse :	5'- ACCATCGCTATCTGAGCAGC-3'
<i>Bcl-2</i>	Forward :	5'- CGACTTCGCCGAGATGCCAGCCAG -3'
	Reverse :	5'- ACTTGTGGCCCAGATAGGCACCCAG -3'
<i>Caspase-8</i>	Forward :	5'- CATCCAGTCACTTGCCAGA -3'
	Reverse :	5'- GCATCTGTTCCCCATGTTT-3'
<i>Bax</i>	Forward :	5'- CCCTTTGCTTCAGGGTTTC -3'
	Reverse :	5'- ACAAAAGTAGAAAAGGGCGACAA -3'
<i>β-Actin</i>	Forward :	5'- GATCATTGCTCCTGAGC -3'
	Reverse :	5'- AAAAGCCATGCCAATCTCATC -3'

(Source: Huang et al. [17]; Quispe-Soto dan Gloria [18]; Quisbert-Valenzuela and Gloria [19]

β-Actin was used as an internal control (housekeeping gene), which was amplified in each reaction. The PCR reactions were performed in a final volume of 50 μL, containing 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.4 M of each oligonucleotide primer, and 2.5 U of Taq DNA polymerase. The PCR was run for 35 cycles for all targets; denaturation in 95 °C for 60 seconds; annealing in 69 °C (p53), 58 °C (Bcl-2, Bax), 56 °C (Caspase-8), and 58 °C (β-Actin) for 60 seconds; and extension in 72 °C for 80 seconds (Kaabinejadian et al., 2008 with modifications). In each PCR cycle, an initial temperature of 94 °C for 10 min was required to activate the Taq polymerase. A final cycle of 72 °C for 5 minutes was used to complete the resulting amplicon [20].

Quantification of qPCR results was carried out using the 2<sup>-ΔΔCt</sup> method. The 2<sup>-ΔΔCt</sup> method is most commonly used for the relative quantification of qPCR [21]. The following is the calculation of the relative differences in gene expression using the 2<sup>-ΔΔCt</sup> method:

Relative fold changes in gene expression = 2<sup>-ΔΔCt</sup>,

where ΔΔCt = ΔCt treated samples – ΔCt untreated samples,

and ΔCt = (Ct target genes – Ct reference targets).

### Mechanism-based yeast bioassay

The Mechanism-based Yeast Bioassay screening method was adapted from

Gunatilaka et al. (1992) and Gunatilaka and Kingston (1998) in Zuhrotun [22]. In this study, wild-type and mutant *Saccharomyces cerevisiae* were used, consisting of *Saccharomyces cerevisiae* strain 1140 (permeable to topoisomerase inhibitor I and has topoisomerase I and II enzymes), 1353 (Rad52 deletion enzyme topoisomerase I (rad52.topI type); only has topoisomerase II enzyme), and 1138 (Rad52 deficient) which is a collection of the Faculty of Pharmacy, Padjadjaran University.

The sample was a mixture of a Ramie leaf extract fraction suspended in methanol-DMSO (1:1). Inoculum of wild-type and mutant yeast strains totaling 10<sup>4</sup> CFU was prepared in a Yeast Potato Dextrose Agar (YPDB) medium and incubated for 24–36 hours at 25–30 °C. The yeast-based bioassay anticancer screening testing technique follows the agar diffusion method of Zuhrotun et al. [10]. This can be divided into subsections if several methods are described [2].

## Results and Discussions

### Phytochemical content of the ramie leaf ethanol extract and its fractions

The results of qualitative phytochemical tests on the ethanol extract of Ramie (*B. nivea* L. Gaud.) leaves and their fractions are presented in Table 2.

**Table 2. Phytochemical Content of the Ramie (*Boehmeria nivea L. Gaud*) the Leaf Ethanol Extract and Its Fractions**

Phytochemical Test	Reagent	Result (+/-)					
		EtOH	n-Heksan	CH <sub>2</sub> Cl <sub>2</sub>	EA	BuOH	H <sub>2</sub> O
Flavonoid	HCl 2N dan Bubuk Mg	-	-	-	-	-	-
Alkaloid	<i>Hager</i>	-	-	-	-	-	-
	<i>Dragendorff</i>	+	-	+	+	+	-
Steroid	<i>Liebermann-Burchard</i>	+	+	+	+	-	-
Triterpenoid	<i>Liebermann-Burchard</i>	-	-	-	-	-	-
Saponin	Hot Water	-	-	-	-	-	-

The steroidal ethanol extract of Ramie leaves was positive with the Dragendorff reagent (in the fractions of ethanol, dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), ethyl acetate (EA), and n-butanol (n-BuOH)) and with the Liebermann-Burchard reagent (in the fractions of ethanol, n-hexane, CH<sub>2</sub>Cl<sub>2</sub>, and EA). Meanwhile, the results of other phytochemical tests (flavonoids, alkaloids, triterpenoids, and saponins) were negative in all fractions (Table 2). Another plant from the same genus, *B. siamensis*, was reported to contain an alkaloid of Boehmeriacin-A that has cytotoxic activity against 12 cancer cell lines and a higher level of anticancer activity compared to standard anticancer drugs [23,24]. Awad et al. [25] also suggested that steroidal-sitosterol compounds from the genus Boehmeria can prevent breast cancer and inhibit the growth of certain types of tumor cells in vitro and reduce the size and rate of tumor metastasis in vivo. Based on this, it is suspected that the Remie leaf extract can be used as an anticancer agent.

#### Cytotoxicity of the remie leaf extract and its fractions on the MCF-7 cell line

The in vitro cytotoxic activity of the Ramie leaf extract and its fractions on the MCF-7 cell line was evaluated using the MTT assay. The cells were incubated for 48 hours with each extract and fraction with the concentrations of 0.1, 1, 10, 100, and 1,000 µg/mL, calculated from the dose-response curve, to determine the IC<sub>50</sub> value (extract concentration that reduced the number of living cells by 50%).

In the treatment of the Ramie leaf ethanol extract cytotoxicity assays were performed on HaCat normal cell lines resulted in IC<sub>50</sub> values 1164.66 + 0.071. However in its fractions, the

CH<sub>2</sub>Cl<sub>2</sub> fraction has the highest cytotoxicity against MCF-7 cells as it produced the lowest IC<sub>50</sub> value (3.79 ± 0.037) µg/mL (Table 3). The steroids contained in the CH<sub>2</sub>Cl<sub>2</sub> fraction (Table 2) can prevent breast cancer and inhibit the growth of several types of specific tumor cells in vitro [25]. The results of this study indicate that this fraction can significantly inhibit the viability of cancer cells and has the potential to have anticancer activity.

**Table 3. The IC<sub>50</sub> Values of the Ramie Leaf Extract and Its Fractions on MCF-7 Cells**

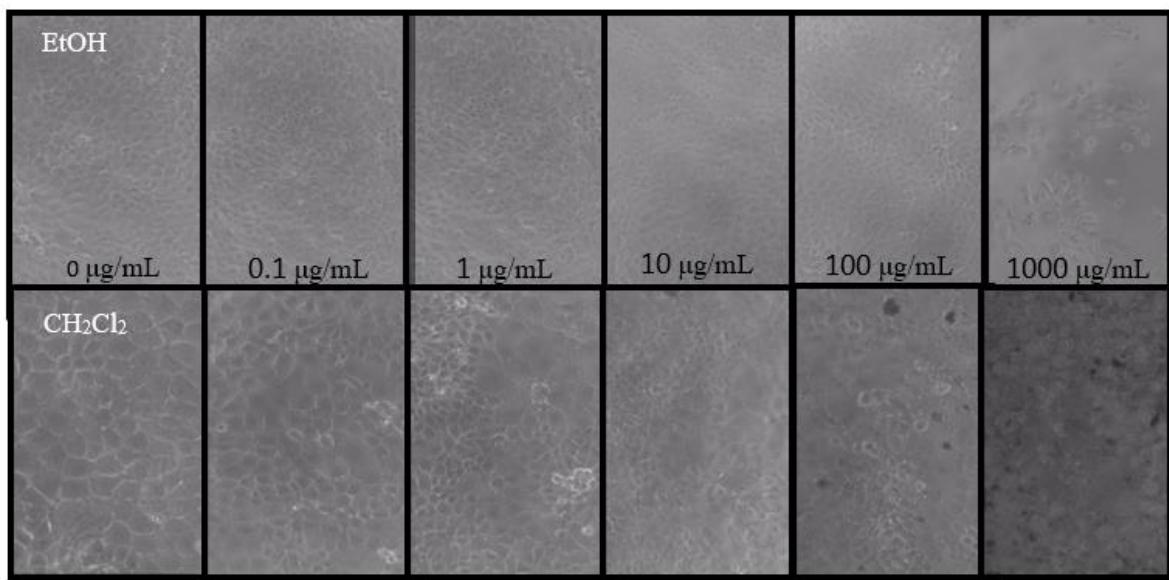
Extract/Fraction	IC <sub>50</sub> (µg/mL)
EtOH (extract)	13.27 ± 0.050
n-Heksana	124.45 ± 0.043
CH <sub>2</sub> Cl <sub>2</sub>	3.79 ± 0.037
EtOAc	156.31 ± 0.033
n-BuOH	431.52 ± 0.068
H <sub>2</sub> O	731.14 ± 0.025

IC<sub>50</sub> ± SD (n = 3)

These results support the study on several genera Boehmeria, which have been reported to produce various compounds that have cytotoxic activity and play a role in the mechanism of apoptosis induction as anticancer agents. Hasibuan et al. [26] stated that β-sitosterol compounds showed the inhibition of T47D and MCF-7 breast cancer cell growth with IC<sub>50</sub> values of 0.55 and 0.87 mM, respectively. In vitro experiments showed that quercetin significantly inhibited the growth of cancer cell cultures with IC<sub>50</sub> values ranging from 7 nM to more than 100 M [27]. This compound can also increase the effectiveness of chemotherapeutic agents [28].

According to the NCI, the IC<sub>50</sub> values can be categorized into strong anticancer potential (<30 µg/mL) and moderately active anticancer potential (30–100 µg/mL). The ethanol extract and the CH<sub>2</sub>Cl<sub>2</sub> fraction of Ramie leaves were included in the active and strong anticancer cate-

gory. This cytotoxicity effect was also confirmed by observing the morphology changes in the MCF-7 cells after they had been treated with the ethanol extract and fractions of Ramie leaves (Figure 1).



*Figure 1. Morphological changes of MCF-7 cells after they had been treated with an EtOH extract and CH<sub>2</sub>Cl<sub>2</sub> fraction of *B. nivea* L. Gaud. with the lowest IC<sub>50</sub> values (concentrations 0, 0.1, 1, 10, 100, and 1,000 µg/mL) after 48 hours (200× magnification)*

The cytotoxicity of several herbal plant extracts on MCF-7 cell proliferation using the MTT test (Table 4) was determined for comparison. The CH<sub>2</sub>Cl<sub>2</sub> fraction of *B. nivea* L. has the smallest IC<sub>50</sub> value compared to other herbal plant extracts. This indicates that the CH<sub>2</sub>Cl<sub>2</sub> fraction has superior anticancer potential as a candidate for a breast cancer drug.

#### ***Apoptosis induction by the active fraction of the ramie leaf extract (*B. Nivea* (L.) Gaud.) against the MCF-7 breast cancer cell line***

The flow cytometry method can quantitatively confirm the cause of cell death by apoptosis or necrosis. It also can distinguish live cells, early apoptosis, late apoptosis, and

necrosis, as Annexin V and PI reagents work selectively in binding intact and non-intact cells (fragmentation). Flow cytometry analysis on MCF-7 cells indicated that in the treatment of the CH<sub>2</sub>Cl<sub>2</sub> fraction, the cell population tended to shift from living cells to apoptotic cells.

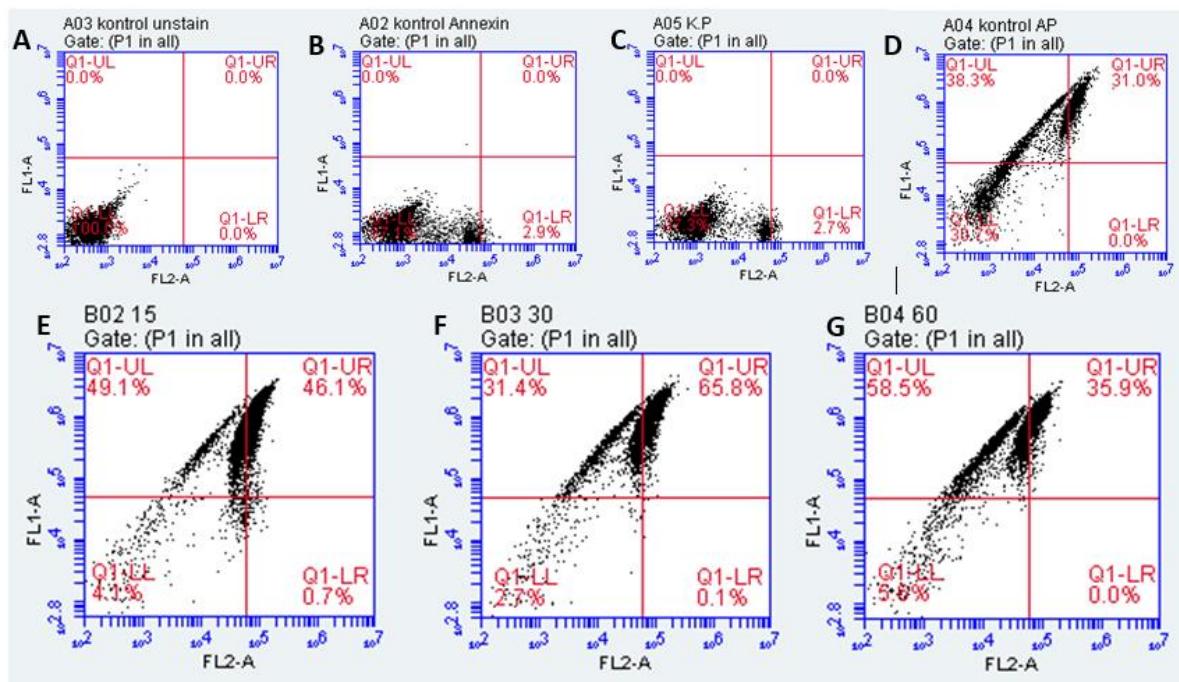
The cytotoxic activity of the active fraction correlated with its ability to induce apoptosis or inhibit the cell cycle. The observations of apoptosis (Figure 2) using flow cytometry indicated that more than 90% of the control cell population (Unstain, Annexin, and PI) had no cell death (all live cells); except for the control Annexin-PI, the living cell population was 30.37%.

Table 4. IC<sub>50</sub> Values of Herbal Plant Extracts on the MCF-7 Cell Line

Species	IC <sub>50</sub> Value (µg/mL)							Reference
	EtOH	MeOH	n-Heksan	CH <sub>2</sub> Cl <sub>2</sub>	EtOAc	n-BuOH	H <sub>2</sub> O	
<i>Boehmeria nivea</i> L. Gaud	13.27 ± 0.050	-	124.45 ± 0.043	3.79 ± 0.037	156.31 ± 0.033	431.52 ± 0.068	731.14 ± 0.025	This study
<i>Piper cubeba</i>	-	22.31 ± 0.83	-	62.20 ± 0.55	-	-	-	Graidist et al. [29]
<i>Ardisia crispa</i>		57.35 ± 19.33	-	-	54.98 ± 14.10	-	>1,000	Nordin et al. [30]
<i>Cyperus longus</i>	-	64.64 ± 1.64	-	25.34 ± 2.01	35.2 ± 2.69	-	-	Memariani et al. [31]
<i>Piper nigrum</i> L.	-	20.25 ± 0.01	-	23.46 ± 1.10	-	-	-	Sriwiri-yajan et al. [32]
<i>Piper retrofractum</i> Vahl	-	19.69 ± 0.88	-	20.03 ± 2.85	-	-	-	
<i>Piper ribesoides</i> Wall.	-	32.27 ± 0.46	-	>80	-	-	-	
<i>Piper betle</i> L.	-	19.30 ± 1.03	-	34.33 ± 1.25	-	-	-	
<i>Piper sarmentosum</i> Roxb.	-	>80	-	>80	-	-	-	
<i>Piper cubeba</i> L.	-	26.63 ± 0.47	-	64.41 ± 1.61	-	-	-	
<i>Piper porphyrophyl-lum</i> N.E.Br.	-	>80	-	>80	-	-	-	
<i>Nardostachys jatamansi</i>	-	58.01 ± 6.13	-	-	65.44 ± 4.63	-	141.35 ± 13.35	Chaudhary et al. [33]
<i>Allium bakhti-aricum</i>	40 ± 1,060	60 ± 1,060	>250	-	60 ± 1,080	-	-	Vafaee et al. [34]
<i>Anona muri-cata</i> Linn	14.68	-	-	-	-	-	538.22	Endrini et al. [35]
<i>Hedyotis corymbosa</i> (L.) Lam.	52.33	-	-	-	-	-	475.71	
<i>Allium sativum</i>	-	-	-	46.94	21.32	61.07	-	Shaban et al. [36]
<i>Moringa oleifera</i> L.	94.44	-	97.60	-	-	-	-	Masfria et al. [37]
<i>Scrophularia umbrosa</i> Dumort	-	-	-	159 ± 2.7	>300	>300	>300	Mansouri et al. [38]

Exposure to the 1/2 IC<sub>50</sub> of the CH<sub>2</sub>Cl<sub>2</sub> fraction resulted in 49.06% of the cell population observed to be in the early stage of apoptosis, followed by 31.43% and 58.55% of the cell population in the same stage after treated with the concentration of IC<sub>50</sub> and 3/2 IC<sub>50</sub> CH<sub>2</sub>Cl<sub>2</sub>, respectively. Meanwhile, the percentage of cells

undergoing necrosis was 0.69% in 1/2 IC<sub>50</sub> concentration, 0.08% in IC<sub>50</sub> concentration, and 0% in 3/2 IC<sub>50</sub> concentration. This proves that the CH<sub>2</sub>Cl<sub>2</sub> fraction of the Ramie leaf extract can induce more than 40% apoptosis in the MCF-7 breast cancer cell line.



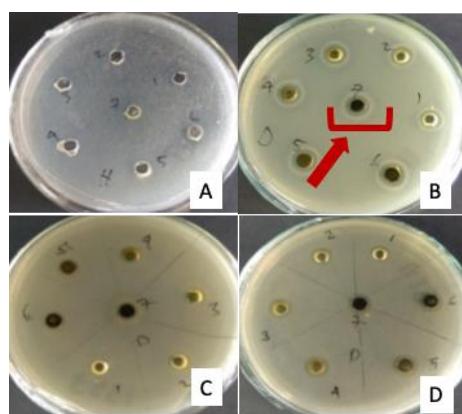
**Figure 2.** Flow cytometric profile of cells with (A) unstained control (without reagents); (B) Annexin control, (C) PI control (K.P); and (D) Annexin-PI control (K. AP); and (E) fraction concentrations of 1/2 IC<sub>50</sub>, (F) IC<sub>50</sub>, (G) 3/2 IC<sub>50</sub>. There are four quadrants: bottom left (Q1-LL) showing viable cells, bottom right (Q1-LR) showing necrotic cells, top left (Q1-UL) showing early apoptotic cells, and top right (Q1-UR) showing late apoptotic cells.

#### **Topoisomerase inhibitor activity of the ramie leaf extract and its fractions using a mechanism-based yeast bioassay**

The active fractions of the ethanol extract were tested for topoisomerase inhibitor activity using the yeast-based bioassay method. Wild-type *S. cerevisiae* was used as a control to ensure that the tested sample had no DNA repair activity. Meanwhile, *S. cerevisiae* strains 1140, 1353, and 1138 indicated the presence of inhibition zones, especially in the CH<sub>2</sub>Cl<sub>2</sub> fraction (Figure 3).

The topoisomerase inhibitor activity was calculated based on the IC<sub>12</sub> value. The fraction is considered active if it shows selective activity against one or more strains and has an IC<sub>12</sub> value < 8,000 µg/mL (Gunatilaka et al., 1998; Gunatilaka et al., 1992; Subong and Primavera, 2012) in Zuhrotun et al. [10]. In this study, the active samples that have the potential as topoisomerase inhibitors are the n-hexane fraction, the CH<sub>2</sub>Cl<sub>2</sub> fraction, and the EtOAc fraction (Table 5).

The Ramie leaf ethanol extract, n-hexane fraction, and EtOAc fraction showed the cell inhibition mechanism through topoisomerase I, whereas the CH<sub>2</sub>Cl<sub>2</sub> fraction showed inhibition through the activity of topoisomerase I and II.



**Figure 3.** Zones of inhibition on *S. cereviseae*: (A) wild-type, (B) strain 1140, (C) strain 1353, and (D) strain 1138 treated with CH<sub>2</sub>Cl<sub>2</sub> fraction concentrations of (1) 125, (2) 250, (3) 500, (4) 1,000, (5) 2,000, (6) 4,000, and (7) 8,000 µg/mL

**Table 5.** IC<sub>12</sub> Values of the Ramie Leaf Extract and Its Fractions on *S. cerevisiae*

Sample	IC <sub>12</sub> (µg/mL)			
	SC	SC 1140	SC 1353	SC 1138
EtOH (extract)	>8,000	4,364.62 ± 909.07	>8,000	>8,000
n-Heksan	>8,000	7,573.48 ± 694.64	>8,000	>8,000
CH <sub>2</sub> Cl <sub>2</sub>	>8,000	231.07 ± 66.39	5,149.42 ± 445.10	518.29 ± 134.31
EtOAc	>8,000	5,782.00 ± 866.32	>8,000	6,394.05 ± 816.05
BuOH	>8,000	>8,000	>8,000	>8,000
H <sub>2</sub> O	>8,000	>8,000	>8,000	>8,000

SC: *Saccharomyces cerevisiae*

Based on Table 5 and Figure 3, the Ramie (*B. nivea* L. Gaud.) leaf extract and its active fraction have an inhibitory mechanism of topoisomerase I and II enzymes. Pommier Y et al. (2010), in Zuhrotun [19], explained that DNA topoisomerase is an important target of anticancer and antibacterial drugs. Ferguson and Baguley (1996), in Zuhrotun [22], also stated that most topoisomerase I and II inhibitors cause mutagenic events related to the replication process and part in cell apoptosis.

The most active fraction as topoisomerase I and II inhibitor was the dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) fraction, which was also the most active fraction for having cytotoxic activity against the MCF-7 cell line with the lowest IC<sub>50</sub> value compared to other fractions. The CH<sub>2</sub>Cl<sub>2</sub> fraction of the Ramie leaf extract can be used as a potential apoptosis-inducing agent, as the phytochemical results indicated that it contains alkaloids and steroids.

#### **Expression of P53, Bcl-2, Bax, and caspase-8 after being exposed to the ramie leaf extract active fraction on the MCF-7 breast cancer cell line**

Apoptosis or programmed cell death is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. There are two main pathways related to gene regulation in apoptosis. First, the extrinsic or cytoplasmic pathway is triggered through the First Apoptotic Signal receptor. This pathway involves the activation of caspases-8 and -10, both of which transmit and amplify the death signal, either through direct activation of effector caspase-3, -6, and -7 or by promoting the Bid involvement of Bax and Bak to activate the intrinsic apoptotic pathway [39].

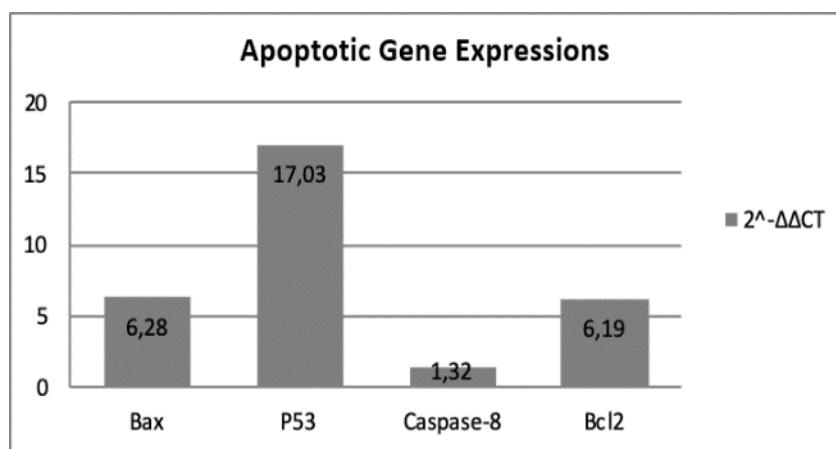
Second, the intrinsic or mitochondrial pathway leads to the release of cytochrome-c from the mitochondria and activation of the death signal. This pathway is tightly regulated by a group of Bcl-2 proteins. There are two main groups of Bcl-2 proteins, namely pro-apoptotic proteins (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk) and anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1) (Reed et al., 1997) in Wong [40]. This pathway also involves the activation of caspase-9 and then activates executioner caspases, namely caspase-3 and -7, to carry out cell death within minutes [39]. The two pathways converge into a series of caspase activation that cleaves regulatory and structural molecules and then ends in cell death. Dysregulation of this pathway often contributes to cancer development and resistance to cancer therapy [39].

In gene expression, the transcription factor that regulates downstream genes that are important in the mechanism of apoptosis is the p53 gene. Loss of p53 in many cancers leads to genomic instability, impaired cell cycle regulation, and inhibition of apoptosis. After DNA damage occurs, p53 holds the cell at the checkpoint until the damage is repaired. If the damage is irreversible, apoptosis cannot be initiated [41]. It can also control the transcription of the Bcl-2 family proteins, especially Bcl-2 and Bax. Also, p53 can activate p21 transcription during DNA damage, which can influence cell cycle progression by interacting with various transcription factors and leading to apoptosis (Gartel et al., 2002; Piccolo et al., 2012) in Yin Sim Tor et al. [42].

The quantity of p53, Bcl-2, Bax, Caspase-8, and β-Actin gene expression induced by the active fraction of the Ramie leaf extract (CH<sub>2</sub>Cl<sub>2</sub>)

was assessed using qPCR. The CH<sub>2</sub>Cl<sub>2</sub> fraction increased the p53 gene activity in MCF-7 cells

up to 17.03 times compared to β-Actin (Figure 4).



**Figure 4.** The Calculation Results of Apoptotic Gene Expression Quantification Using the Livak ( $2^{-\Delta\Delta CT}$ ) Method

This study demonstrated that the CH<sub>2</sub>Cl<sub>2</sub> fraction of the Ramie leaf extract induced apoptosis in MCF-7 cells through an intrinsic mechanism of gene damage involving the p53 gene. The p53 protein functions as transcription factors that regulate downstream genes that are important in cell cycle arrest, DNA repair, and apoptosis. It also controls the transcription of the Bcl-2 family, especially Bcl-2 and Bax (Garrett et al., 2002; Piccolo et al., 2012) in Yin Sim Tor et al. [42]. Ramie leaves (*B. nivea* L. Gaud.) have the potential to be an alternative drug source for breast cancer therapy.

## Conclusion

The CH<sub>2</sub>Cl<sub>2</sub> fraction of Ramie leaves can inhibit the proliferation of MCF-7 cells *in vitro* with an IC<sub>50</sub> value of 3.27 µg/mL, which is included in the active and strong anticancer category. The CH<sub>2</sub>Cl<sub>2</sub> fraction could actively induce more than 40% apoptosis in the MCF-7 cells. The CH<sub>2</sub>Cl<sub>2</sub> fraction induces apoptosis by modulating the expression levels of Bax, Bcl-2, and p53 genes. The highest gene expression level was in p53, which was 17.03 times compared to that due to β-Actin. The CH<sub>2</sub>Cl<sub>2</sub> fraction can also inhibit topoisomerase I and topoisomerase II, which was first reported in this study. These results showed the potential of Ramie leaves to be developed as an anticancer drug candidate.

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