Anticancer Effects of Vitex trifolia Extract on Cervical Cancer Animal Models: A Study of Genetic Mutations and Anti-Apoptotic Bcl-2 Gene Expression

Humairah Medina Liza Lubis*[1], Emni Purwoningsih[2], and Anggi Yulisna Frisilia Saputri[3]

[1] Department of Anatomical Pathology, Faculty of Medicine Universitas Muhammadiyah Sumatera Utara, Jl Gedung Arca No 53 – 20217, Medan, Indonesia
[2] Department, Muhammadiyah University of North Sumatera, Medan, Indonesia
[3] Faculty of Medicine Universitas Muhammadiyah Sumatera Utara, Jl Gedung Arca No 53 – 20217, Medan, Indonesia
humairahmedina@umsu.ac.id

Abstract. This study investigates the potential of Vitex trifolia (legundi) extract to reduce resistance to radiation and chemotherapy in epithelial cancer by targeting the Bcl-2 apoptosis pathway. The objectives include (1) characterizing Casticin and Labdane-Type Diterpene compounds; (2) analyzing Bcl-2 gene mutations in chemotherapy resistance; (3) studying molecular docking interactions with Bcl-2 protein; (4) evaluating histopathological effects in cervical cancer animal models; (5) assessing apoptosis via Bcl-2 expression changes; and (6) ensuring safety through liver and kidney function tests. Using in vitro and in vivo methods, the study employed phytochemical screening, molecular docking, and a rat cervical cancer model treated with Vitex trifolia extract. GC-MS confirmed the presence of Casticin and Labdane-Type Diterpene. Molecular docking revealed strong interactions between Labdane-Type Diterpene and Bcl-2. Histopathological improvements and reduced Bcl-2 expression were observed, suggesting Vitex trifolia extract could enhance cancer treatment efficacy by overcoming chemotherapy resistance.

Keywords: Vitex trifolia; Cervical Cancer; Genetic Mutations; Anti-Apoptotic Bcl-2; Resistance.

1 Introduction

Cervical cancer is the fourth most common cancer in women, with 660,000 new cases reported in 2022. Approximately 94% of the 350,000 annual deaths caused by cervical cancer occur despite the use of adequate therapy, with therapy failure still being observed. The incidence of local recurrence is about 10-20% in early-stage cervical cancer and 40-60% in advanced stages. Cancer cells can develop resistance to radiation and chemotherapy, with one of the mechanisms contributing to this resistance being the inhibition of apoptosis.

E. Fachrial and S. Suhartomi (eds.), *Proceedings of the 1st International Conference on Lifestyle Diseases and Natural Medicine (ICOLIFEMED 2024)*, Advances in Health Sciences Research 84,
https://doi.org/10.2991/978-94-6463-664-2_19

Human Papillomavirus (HPV) infection, a leading cause of cervical cancer, can affect human genes, particularly those involved in apoptosis, cell cycle, and proliferation. The integration of HPV DNA into the genome of cervical epithelial cells leads to the persistence of viral oncogenes E6 and E7, resulting in uncontrolled proliferation and carcinogenesis. In cancer cells, genetic instability and mutations in apoptosis-related genes, including Bcl-2, lead to increased expression of anti-apoptotic Bcl-2 protein, which is a marker of neoplastic progression. In patients with primary and recurrent cervical cancer, positive staining for anti-apoptotic Bcl-2 is associated with poorer 5-year survival rates.

The issue of therapy resistance can be minimized using natural phytochemicals, although information on this is still limited. One natural agent with proven anticancer effects is Vitex trifolia (legundi). Ethanol extracts of legundi fruit have shown the ability to inhibit proliferation activity, tumor growth, and p53 expression in skin cancer. Flavonoids in legundi leaf extract, such as Casticin, have been reported to inhibit cancer cell proliferation in rats and activate apoptosis. Additionally, rotundifuran (a Labdane-type diterpene) from Legundi has been shown to suppress cervical cancer proliferation in HeLa and SiHa cells. Research on Casticin and Labdane-Type Diterpene from Legundi in cervical cancer is still limited, indicating a need for further study. These compounds could potentially be considered adjunct therapies to improve survival rates.

The specific objectives of this study are: (1) To analyze the characterization of Casticin and Labdane-Type Diterpene compounds from legundi fruit; (2) To investigate the mechanism of anti-apoptotic Bcl-2 gene mutations and chemotherapy resistance in cervical cancer; (3) To analyze the molecular docking interaction between Casticin and Labdane-Type Diterpene compounds and the anti-apoptotic Bcl-2 protein; (4) To evaluate the effects of Casticin and Labdane-Type Diterpene compounds from legundi fruit on the histopathological profiles of cervical tissue in animal models of cervical cancer induced by *benzo*[α]*pyrene*; (5) To assess the activity of these compounds in enhancing apoptosis by altering the expression of Bcl-2 anti-apoptosis markers in cervical cancer animal models; and (6) To analyze the safety of Casticin and Labdane-Type Diterpene administration through liver and kidney function parameters. Based on feasibility studies and in vitro and in vivo research results, legundi has been reported to possess active compounds with potential anticancer properties, forming the basis for further testing its activity against cervical cancer in animal models using apoptosis (Bcl-2) parameters.

2 Literature Review

Legundi (Vitex trifolia) is a small-leaved shrub prevalent in Asian countries, including Indonesia. Modern research has demonstrated that legundi extracts possess cytotoxic properties against several cancer cell lines and exhibit antimicrobial activity. Previous phytochemical studies on legundi fruit have highlighted the presence of diterpenoids, flavonoids, and phenolic acid constituents, some of which have shown significant biological activities, including cytotoxicity against

mammalian cancer cells and inhibition of mosquito-borne diseases. revealed that diterpene compounds could inhibit proliferation, induce apoptosis at higher concentrations, and arrest the cell cycle of tsFT210 and K562 cell lines at the G0/G1 phase at lower concentrations. Labdane-type diterpenes have shown promise as chemotherapy agents, suggesting that legundi may effectively treat certain cancers. However, research on Casticin and Labdane-Type Diterpenes, especially in cervical cancer, remains limited, necessitating further exploration into their roles in apoptosis, cell cycle regulation, and proliferation processes. Cervical Cancer Overview Cervical cancer is the fourth most common cancer among women worldwide. Most cervical cancers are squamous cell carcinomas (SCC) resulting from HPV infections, with HPV types 16 and 18 being the most prevalent. This cancer predominantly affects women of low socioeconomic status, with risky sexual behaviors, such as having multiple sexual partners and not using condoms, being significant risk factors. The average age of diagnosis is around 49 years, and due to subtle early symptoms, cervical cancer is often diagnosed at advanced stages.

Common symptoms include post-coital bleeding, intermenstrual bleeding, increased vaginal discharge, pelvic pain, and lower back pain. Although early-stage cervical cancer can often be cured, treatment-related side effects, such as infertility, are common. The average survival time for patients with metastatic or recurrent cervical cancer is typically less than two years. Apoptosis, Genetic Mutations, and Chemotherapy Resistance The p53 gene and anti-apoptosis protein Bcl-2 play critical roles in apoptosis in normal and tumor cells. Increased expression of anti-apoptotic Bcl-2 impacts caspase function, inhibiting apoptosis. Bcl-2 stabilizes the mitochondrial membrane, preventing the release of apoptosis-inducing factors. However, due to DNA repair mechanisms, chemotherapy resistance is often linked to the cancer cell's limited sensitivity to cytotoxic effects. Dysregulation or damage to DNA repair genes is common in many cancer cells, and Bcl-2 expression levels after radiation therapy serve as valuable prognostic markers for survival rates in cervical cancer patients.

3 Methods

The identification of the Vitex trifolia plant was conducted at the Herbarium Medanese Laboratory (MEDA), University of North Sumatra. A total of 2,500 grams of Vitex trifolia fruit was cleaned, washed, dried, and ground. The dried plant material was then soaked in 96% ethanol (1:10) for 3 days with occasional stirring, stored in a dark place. It was filtered using vacuum filtration and Whatman filter paper no. 40, then evaporated using a rotary evaporator at T=55°C and P=80 mBar.

Next, a comprehensive phytochemical screening of the ethanol extract was conducted, both qualitatively and quantitatively, using advanced techniques such as Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrophotometry (GC-MS) to identify its compounds. Additionally, total flavonoids, phenols, Casticin, and Labdane-Type Diterpenes were measured.

This screening provided a detailed chemical profile of the extract, allowing for a better understanding of its potential bioactive components. The final steps is to investigate molecular docking with protein ligands using silica analysis.

Following this, molecular docking studies were performed on Casticin and Labdane-Type Diterpenes to predict their interactions with target proteins. This step utilized software tools such as Autodocktool and Pymol, providing insights into the possible mechanisms of action of the compounds at the molecular level. The molecular docking results were essential for selecting the compounds for further biological testing.

To assess the anticancer potential of the extract, a cervical cancer model was developed using Rattus norvegicus rats, induced with benzo(a)pyrene. The induction process involved the application of benzo(a)pyrene to the cervical surface, and the development of cancerous cells was confirmed via Pap smear tests. Subsequently, the rats were treated with varying doses of the legundi extract. The creation of a reliable animal model was crucial for evaluating the therapeutic effects of the extract in a controlled environment.

Histopathological examinations were then conducted to determine the type of cervical cancer developed in the animal models. Histopathological examination was performed using Hematoxylin and Eosin (HE) staining to determine the type of cancer, including keratinizing squamous cell carcinoma, non-keratinizing squamous cell carcinoma, adenocarcinoma, and adenosquamous carcinoma. Additionally, an immunohistochemical examination for the anti-apoptosis protein Bcl-2 was conducted. Before staining, xylene was removed from the tissue sections, and the samples were rehydrated through a series of graded ethanol. The antibody used was Bcl-2 from RD Systems (dilution 1:200). Immunohistochemical (IHC) staining was performed using Cell Conditioning 1 Solution for antigen retrieval and the OptiView DAB IHC Detection Kit (Ventana). Non-immune serum was replaced with the primary antibody as a negative control, while positive control was skin tissue previously known to be positive for Bcl-2. Quantitative assessment was performed visually using a light microscope at 400x magnification, evaluating immunoreactive cells indicated by a positive expression that appeared as reddish-brown staining in the cytoplasm. The number of Bcl-2 expressing cells was counted in 10 fields of view, and the examination was conducted by specialist doctors in Anatomical Pathology.

To ensure the safety of the extract, liver and kidney functions were tested by measuring SGOT, SGPT, urea, and creatinine levels in both normal and cancer-induced animals. This testing ensured that the extract did not cause any adverse effects on vital organs, thereby validating its potential as a therapeutic agent.

Finally, the study involved an immunohistochemistry examination of anti-apoptotic Bcl-2 expression. The expression of Bcl-2 was assessed using specific staining techniques, and the results were quantitatively analyzed under a microscope. This step helped confirm the mechanism by which the extract influences cancer cell survival, providing a deeper understanding of its anticancer properties.

This research received an ethical approval letter from the Health Ethics Committee of the Faculty of Medicine, Universitas Muhammadiyah Sumatera Utara, with the ethical approval description No: 894/KEPK/FKUMSU/2022.

4 Results

4.1 Phytochemical screening of the 96% ethanol extract was conducted both qualitatively and quantitatively using GC-MS to identify *Casticin* and *Labdane-Type Diterpenes*

The extract of legundi fruit (Vitex trifolia L.) was analyzed using Gas Chromatography Mass Spectrophotometry (GC-MS) to identify its compounds both qualitatively and quantitatively. The chromatogram peak for Casticin, identified as 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one, appears at around 10 minutes. The results are shown in Figures 1 and 2 below.

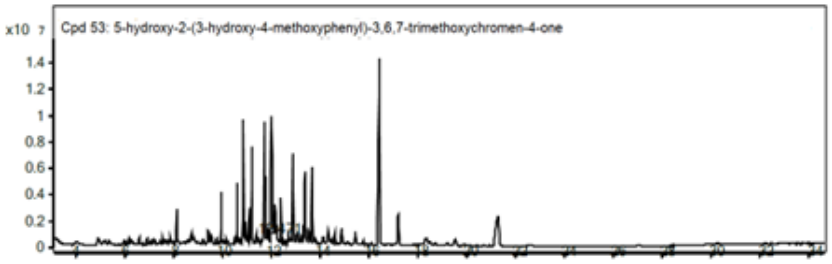


Fig. 1. GC-MS Results for Casticin in Legundi Fruit (Vitex trifolia L.) Extract

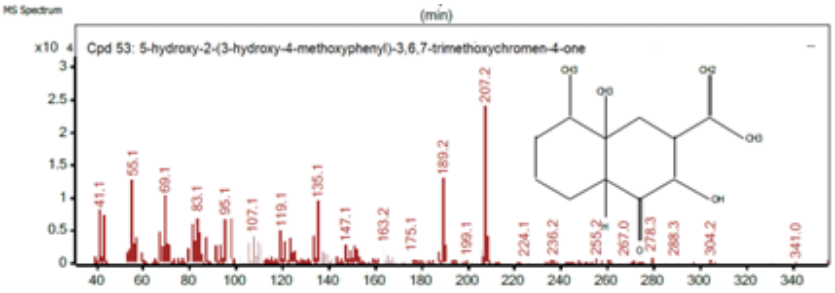


Fig. 2. GC-MS Results for Casticin in Legundi Fruit (Vitex trifolia L.) Extract.)

From Figures 1 and 2 above, the compound 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one was identified in the legundi fruit (Vitex trifo-

lia L.). The highest concentration is observed at peak 207.2, with a value of 23,092.46. The total concentrations are presented in Table 2 below

Table 1. Concentrations of 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one identified in legundi fruit extract based on GC-MS data

MS	z	Consentration
41.1	1	7911.53
43.1		7090.96
55.1		12520.96
69.1		10113.09
81.1		5695.46
83.1		6611.15
95.1		6390.22
135.2	1	9380.81
192.2	1	12774.29

The chromatogram peak for Labdane-Type Diterpene, identified as 1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene, appears at around 10 minutes. The results are shown in Figures 3 and 4 below.

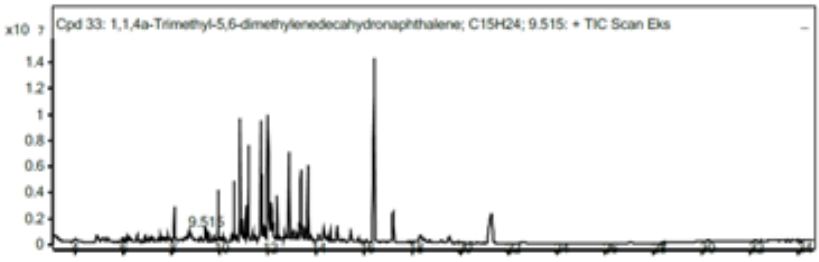


Fig. 3. GC-MS Results for Labdane-Type Diterpene in Legundi Fruit Extract)

From Figures 4 and 5 above, the compound 1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene, with the molecular formula C15H24, was identified in the legundi fruit (Vitex trifolia L.). The highest concentration is observed at peak 43.1, with a value of 41,152.13.

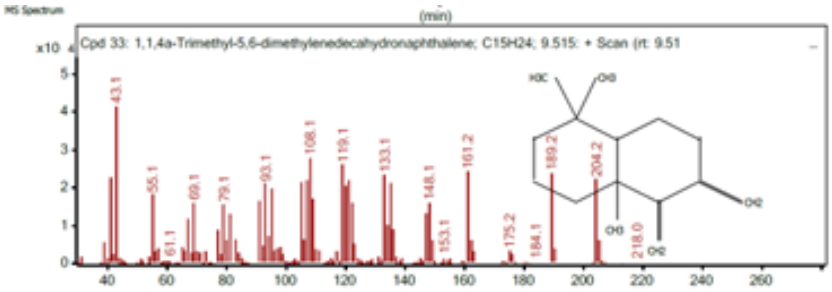


Fig. 4. MS Peak Spectrum of Legundi Fruit (Vitex trifolia L.) Extract

Table 2. Concentrations of 1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene identified in legundi fruit extract based on GC-MS data

MS	z	Concentration
41.1		22184.13
43.1	1	**41184.13**
107.1		21480.77
108.1		17450.42
119.1		25417.16
121.1		21675.61
133.1		22095.91
161.2		23976.27
189.2	1	23278.71
204.2	1	21806.41

4.2 Molecular docking was performed on Casticin and Labdane-Type Diterpene to predict suitable docking sites on the target protein and to analyze protein-ligand interactions

After docking, interactions between the ligands Casticin and Labdane-Type Diterpene with the Bcl-2 protein receptor were observed. Figures 5 and 6 illustrate the interaction and hydrophobic binding between these compounds.

Tables 4 and 5 present the docking results between the receptor and ligands. The findings indicate that Labdane-Type Diterpene exhibits better interaction capabilities with Bcl-2 compared to Casticin. A more negative Gibbs energy suggests a stronger binding interaction between the ligand and receptor. Additionally, smaller inhibition constants indicate stronger ligand-protein binding,

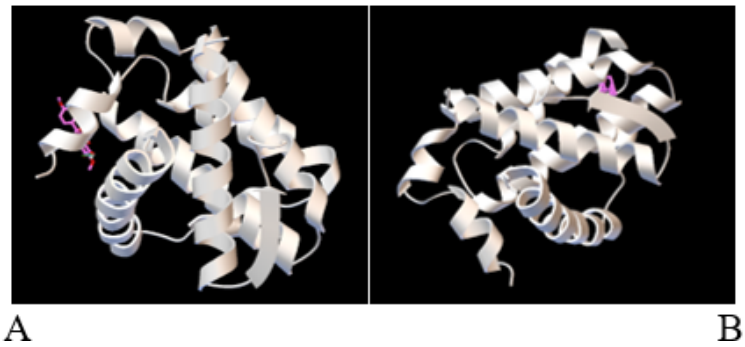


Fig. 5. Interaction of Casticin and Labdane-Type Diterpene Ligands with the Bcl-2 Receptor

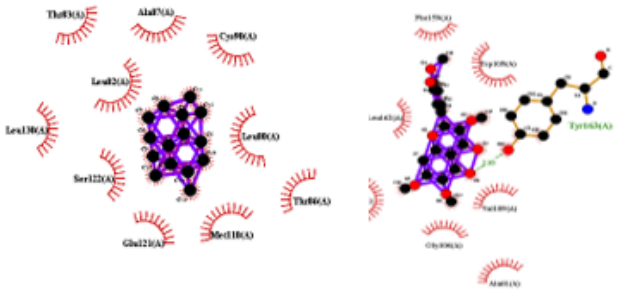


Fig. 6. Hydrophobic Bonding Between Casticin and Labdane-Type Diterpene Ligands with the Bcl-2 Receptor

and a greater number of hydrophobic bonds signify a more robust ligand-protein interaction.

Table 3. Docking Results Between Casticin Ligand and Bcl-2 Receptor

Labdane-type diterpene	**Bcl-2**	
Gibbs Energy		-3.82
pKi		1,59
Hydrogen bonding	1 → Tyr163	
Hydrophobic bonding	7	
Hydrophobic binding residues	Ala61, Arg68,	Trp105, Gly106, Val109, Phe159, Leu162

Table 4. Docking Results Between Labdane-Type Diterpene ligand and Bcl-2 Receptor

Labdane-type diterpene	***Bcl-2***
Gibbs Energy	-7.8
pKi	1.91 μM
Hydrogen bonding	None (−)
Hydrophobic bonding	10
Hydrophobic binding residues	Leu80, Leu82, Thr83, Thr86, Ala87, Cys90, Met118, Glu121, Ser122, Leu130

4.3 Treatment of cervical cancer model animals with benzo(a)pyrene induction

Rattus norvegicus rats were used as test animals, induced with benzo(a)pyrene (50 mg/kg body weight in 0.5 ml corn oil) applied to the cervical surface. To confirm the development of cancer cells, a Pap smear was conducted. After confirmation, the rats were treated with ethanol extract of legundi fruit at doses of 0.1 g/kg, 0.5 g/kg, and 1 g/kg body weight orally for 30 days. The treatment groups consisted of (1) Negative control (normal) group, where the test animals were given only standard feed without any treatment; (2) Positive control group, where the test animals were given benzo(a)pyrene, serving as the cervical cancer

model; and (3) Treatment groups P1, P2, and P3, where the cancer model rats were treated with legundi fruit ethanol extract at doses of 0.1 g/kg, 0.5 g/kg, and 1 g/kg body weight, respectively.

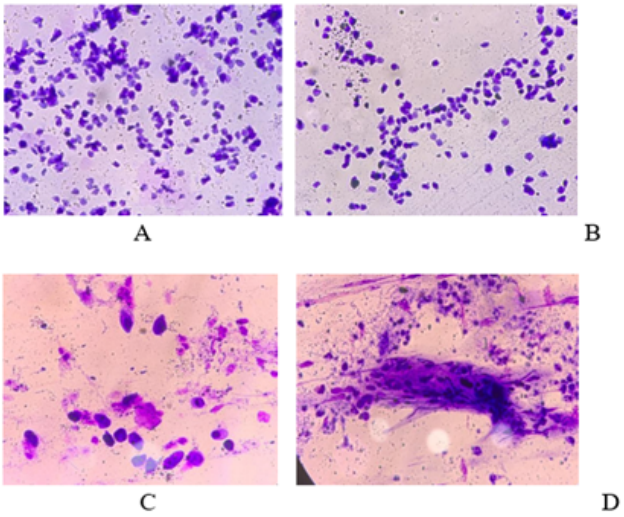


Fig. 7. Cytological Pap Smear Results. A. C2 Benign Smear, B. C3 Atypia Probably Benign, C. C4 Suspicious Malignancy, D. C5 Malignancy

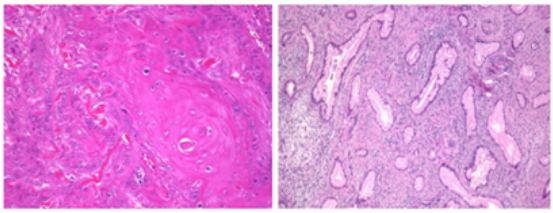
Table 5. Cytological Results of Pap Smear Test in Cervical Cancer Model Rats

Pap smear result	Negative Control	Positive Control	Treatment I	Treatment II	Treatment III	Total
C1 Inadequate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C2 Benign	6 (20%)	0 (0%)	1 (3.3%)	1 (3.3%)	1 (3.3%)	9 (30%)
C3 Atypia probably benign	0 (0%)	1 (3.3%)	1 (3.3%)	1 (3.3%)	0 (0%)	3 (10%)
C4 Suspicious malignancy	0 (0%)	2 (6.7%)	2 (6.7%)	2 (6.7%)	2 (6.7%)	8 (26.7%)
C5 Malignancy	0 (0%)	4 (13.3%)	2 (6.7%)	2 (6.7%)	2 (6.7%)	10 (33.3%)
Total	**6 (20%)**	**6 (20%)**	**6 (20%)**	**6 (20%)**	**6 (20%)**	**30 (100%)**

4.4 Histopathological Examination for Cancer Type Determination

After 30 days of administering legundi fruit ethanol extract (Vitex trifolia), the rats were euthanized, and their cervical organs were harvested for histopatholog-

ical examination to determine the type of cervical cancer. The histopathological images of cervical cancer can be seen in Figure 8 below.



A B

Fig. 8. Histopathological Images of Cervical Cancer. A. Squamous Cell Carcinoma, B. Adenocarcinoma

4.5 Immunohistochemical Examination of Anti-Apoptotic Bcl-2

Immunohistochemical examination of anti-apoptotic Bcl-2 was conducted to assess changes in Bcl-2 expression across the different rat groups. The results of the Bcl-2 immunohistochemical expression can be found in Table 7 and Figure 9 below.

Table 6. Immunohistochemical Expression of Anti-Apoptotic Bcl-2

No	Rats Group	Intensity (I)	Areal (%)
1	K- U1	0	0
2	K- U2	0	0
3	K- U3	0	0
4	K- U4	0	0
5	K- U5	0	0
6	K- U6	0	0
7	K+ U1	+3	80
8	K+ U2	+3	75
9	K+ U3	+3	50
10	K+ U4	+3	75
1	K- U1	0	0
2	K- U2	0	0
3	K- U3	0	0
4	K- U4	0	0
5	K- U5	0	0
6	K- U6	0	0
7	K+ U1	+3	80
8	K+ U2	+3	75
9	K+ U3	+3	50
10	K+ U4	+3	75
11	K+ U5	+3	50
12	K+ U6	+3	50
13	P1 U1	+3	50
14	P1 U2	+3	50
15	P1 U3	+3	50
16	P1 U4	+3	70
17	P1 U5	+3	10
18	P1 U6	+3	20
19	P2 U1	+3	20
20	P2 U2	+3	20
21	P2 U3	+2	75
22	P2 U4	0	0
23	P2 U5	+3	50
24	P2 U6	+3	50
25	P3 U1	+3	50
26	P3 U2	+3	20
27	P3 U3	+3	20
28	P3 U4	+3	75
29	P3 U5	0	0
30	P3 U6	0	0

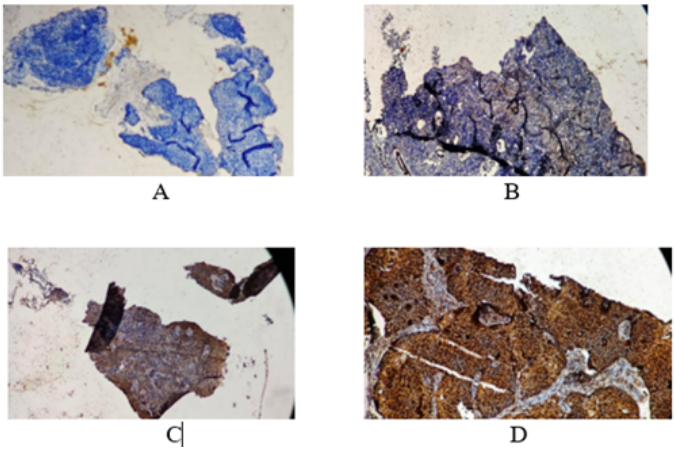


Fig. 9. Intensity of Bcl-2 Expression in Cervical Cancer. A. Score 0 (400x), B. Score 1 (100x), C. Score 2 (100x), D. Score 3 (400x)

4.6 Relationship Between Bcl-2 Expression Intensity in Cervical Cancer and Legundi Fruit (Vitex trifolia L.) Administration

Table 7. *Bcl-2 Expression (Allred score)* in Histopathological Types

Bcl-2 Expression (Allred score)	Normal		Squamous Cell Carcinoma		Adenocarcinoma	
	n	%	n	%	n	%
None-20%	11	36.7	5	16.7	0	0
50-75%	0	0	12	40	2	6.7
¿75%	0	0	1	3.3	0	0
Total	**11**	**36.7**	**18**	**60**	**1**	**3.3**
Total (%)			**30 (100)**			**p-value: 0.035**

**Fisher's exact test: p¡0.05*

SPSS analysis using Fisher's exact test yielded a result of p=0.035 (p¡0.05), indicating a significant relationship between the loss or reduction of Bcl-2 expression in cervical cancer histopathology and the administration of legundi fruit extract.

Bcl-2 stabilizes cell membranes and prevents the release of apoptosis-inducing factors. When the Bcl-2/BAX complex forms, Bcl-2 loses its inhibitory activity. BAX migrates to the mitochondrial membrane upon receiving an apoptotic signal, triggering the release of cytochrome C and AIF into the cytosol. AIF enters the nucleus, causing chromatin condensation and nuclear fragmentation, followed by caspase activation. Bcl-2 plays a key role in radiation sensitivity. Increased

Bcl-2 expression is associated with poorer survival in cervical cancer patients, as Bcl-2-positive tumors show weaker responses to radiation. Bcl-2 expression levels after radiation therapy are useful prognostic markers. Chemotherapy resistance can occur through apoptosis inhibition, often due to mutations in the p53 gene. The ethanol extract of legundi fruit has shown potential in inhibiting tumor growth and p53 expression in skin cancer. Flavonoids like Casticin and Labdane-Type Diterpenes in legundi can inhibit cancer cell proliferation and induce apoptosis. This study suggests that legundi extract may be developed for future therapeutic use by targeting the Bcl-2 apoptosis pathway

4.7 Phytochemical screening of the 96% ethanol extract was conducted both qualitatively and quantitatively using GC-MS to identify *Casticin* and *Labdane-Type Diterpenes*

The extract of legundi fruit (Vitex trifolia L.) was analyzed using Gas Chromatography Mass Spectrophotometry (GC-MS) to identify its compounds both qualitatively and quantitatively. The chromatogram peak for Casticin, identified as 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one, appears at around 10 minutes. The results are shown in Figures 1 and 2 below.

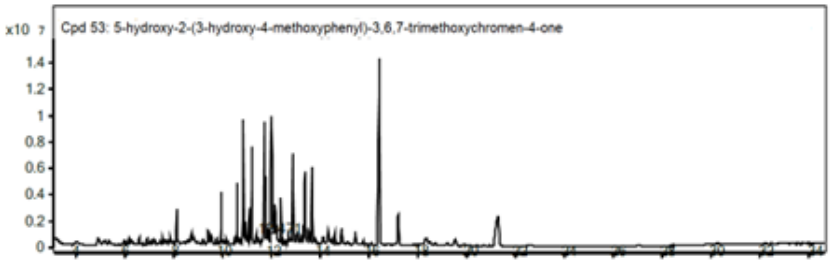


Fig. 10. GC-MS Results for Casticin in Legundi Fruit (Vitex trifolia L.) Extract

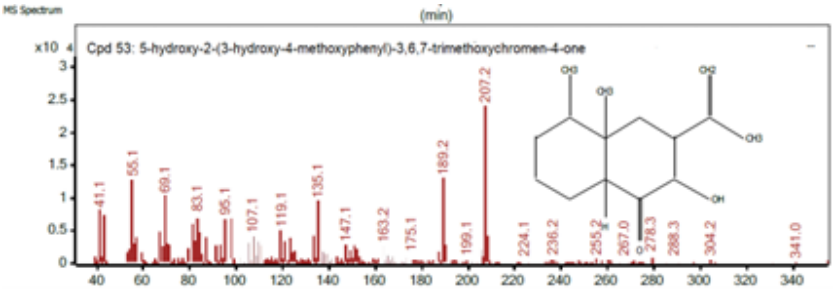


Fig. 11. GC-MS Results for Casticin in Legundi Fruit (Vitex trifolia L.) Extract.)

From Figures above, the compound 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one was identified in the legundi fruit (Vitex trifolia L.). The highest concentration is observed at peak 207.2, with a value of 23,092.46. The total concentrations are presented in Table 2 below

Table 8. Concentrations of 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxychromen-4-one identified in legundi fruit extract based on GC-MS data

MS	z	Consentration
41.1	1	7911.53
43.1		7090.96
55.1		12520.96
69.1		10113.09
81.1		5695.46
83.1		6611.15
95.1		6390.22
135.2	1	9380.81
192.2	1	12774.29

The chromatogram peak for Labdane-Type Diterpene, identified as 1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene, appears at around 10 minutes. The results are shown in Figures 3 and 4 below.

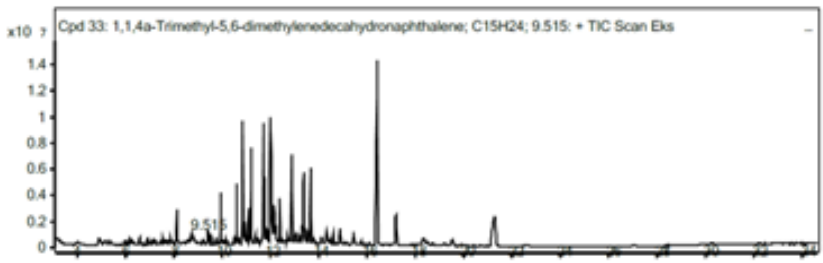


Fig. 12. GC-MS Results for Labdane-Type Diterpene in Legundi Fruit Extract)

From Figures above, the compound 1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthale with the molecular formula C15H24, was identified in the legundi fruit (Vitex

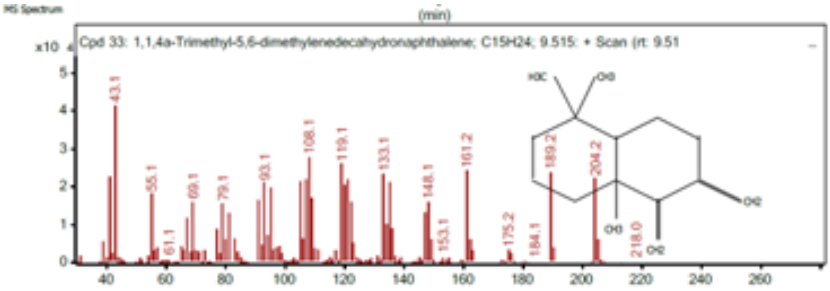


Fig. 13. MS Peak Spectrum of Legundi Fruit (Vitex trifolia L.) Extract

trifolia L.). The highest concentration is observed at peak 43.1, with a value of 41,152.13.

Table 9. Concentrations of 1,1,4a-Trimethyl-5,6-dimethylenedecahydronaphthalene identified in legundi fruit extract based on GC-MS data

MS	z	Concentration
41.1		22184.13
43.1	1	**41184.13**
107.1		21480.77
108.1		17450.42
119.1		25417.16
121.1		21675.61
133.1		22095.91
161.2		23976.27
189.2	1	23278.71
204.2	1	21806.41

4.8 Molecular docking was performed on Casticin and Labdane-Type Diterpene to predict suitable docking sites on the target protein and to analyze protein-ligand interactions

After docking, interactions between the ligands Casticin and Labdane-Type Diterpene with the Bcl-2 protein receptor were observed. Figures 5 and 6 illustrate the interaction and hydrophobic binding between these compounds.

Tables above present the docking results between the receptor and ligands. The findings indicate that Labdane-Type Diterpene exhibits better interaction

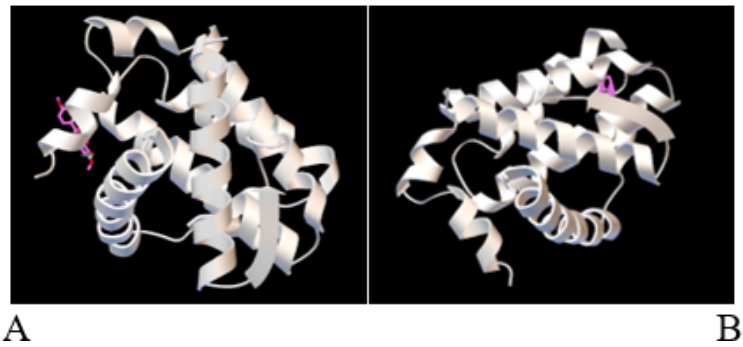


Fig. 14. Interaction of Casticin and Labdane-Type Diterpene Ligands with the Bcl-2 Receptor

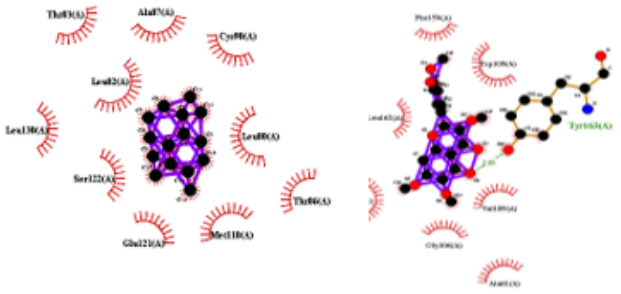


Fig. 15. Hydrophobic Bonding Between Casticin and Labdane-Type Diterpene Ligands with the Bcl-2 Receptor

capabilities with Bcl-2 compared to Casticin. A more negative Gibbs energy suggests a stronger binding interaction between the ligand and receptor. Additionally, smaller inhibition constants indicate stronger ligand-protein binding, and a greater number of hydrophobic bonds signify a more robust ligand-protein interaction.

Table 10. Docking Results Between Casticin Ligand and Bcl-2 Receptor

Labdane-type diterpene	**Bcl-2**	
Gibbs Energy		-3.82
pKi		1,59
Hydrogen bonding	1 → Tyr163	
Hydrophobic bonding	7	
Hydrophobic binding residues	Ala61, Arg68,	Trp105, Gly106, Val109, Phe159, Leu162

Table 11. Docking Results Between Labdane-Type Diterpene ligand and Bcl-2 Receptor

Labdane-type diterpene	***Bcl-2***
Gibbs Energy	-7.8
pKi	1.91 μM
Hydrogen bonding	None (−)
Hydrophobic bonding	10
Hydrophobic binding residues	Leu80, Leu82, Thr83, Thr86, Ala87, Cys90, Met118, Glu121, Ser122, Leu130

4.9 Treatment of cervical cancer model animals with benzo(a)pyrene induction

Rattus norvegicus rats were used as test animals, induced with benzo(a)pyrene (50 mg/kg body weight in 0.5 ml corn oil) applied to the cervical surface. To confirm the development of cancer cells, a Pap smear was conducted. After confirmation, the rats were treated with ethanol extract of legundi fruit at doses

of 0.1 g/kg, 0.5 g/kg, and 1 g/kg body weight orally for 30 days. The treatment groups consisted of (1) Negative control (normal) group, where the test animals were given only standard feed without any treatment; (2) Positive control group, where the test animals were given benzo(a)pyrene, serving as the cervical cancer model; and (3) Treatment groups P1, P2, and P3, where the cancer model rats were treated with legundi fruit ethanol extract at doses of 0.1 g/kg, 0.5 g/kg, and 1 g/kg body weight, respectively.

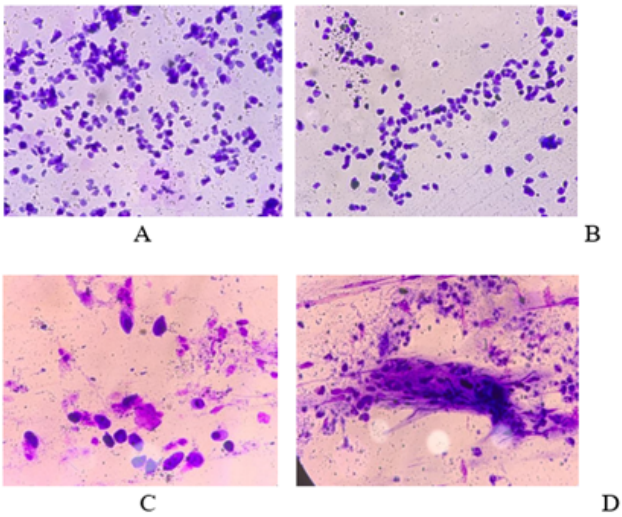


Fig. 16. Cytological Pap Smear Results. A. C2 Benign Smear, B. C3 Atypia Probably Benign, C. C4 Suspicious Malignancy, D. C5 Malignancy

Table 12. Cytological Results of Pap Smear Test in Cervical Cancer Model Rats

Pap smear result	Negative Control	Positive Control	Treatment I	Treatment II	Treatment III	Total
C1 Inadequate	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C2 Benign	6 (20%)	0 (0%)	1 (3.3%)	1 (3.3%)	1 (3.3%)	9 (30%)
C3 Atypia probably benign	0 (0%)	1 (3.3%)	1 (3.3%)	1 (3.3%)	0 (0%)	3 (10%)
C4 Suspicious malignancy	0 (0%)	2 (6.7%)	2 (6.7%)	2 (6.7%)	2 (6.7%)	8 (26.7%)
C5 Malignancy	0 (0%)	4 (13.3%)	2 (6.7%)	2 (6.7%)	2 (6.7%)	10 (33.3%)
Total	**6 (20%)**	**6 (20%)**	**6 (20%)**	**6 (20%)**	**6 (20%)**	**30 (100%)**

4.10 Histopathological Examination for Cancer Type Determination

After 30 days of administering legundi fruit ethanol extract (Vitex trifolia), the rats were euthanized, and their cervical organs were harvested for histopathological examination to determine the type of cervical cancer. The histopathological images of cervical cancer can be seen in Figure 8 below.

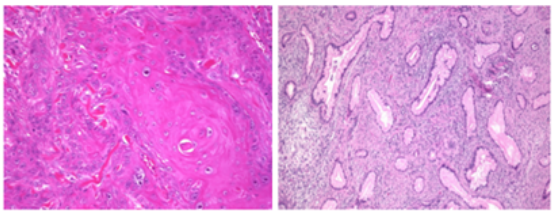


Fig. 17. Histopathological Images of Cervical Cancer. A. Squamous Cell Carcinoma, B. Adenocarcinoma

4.11 Immunohistochemical Examination of Anti-Apoptotic Bcl-2

Immunohistochemical examination of anti-apoptotic Bcl-2 was conducted to assess changes in Bcl-2 expression across the different rat groups. The results of the Bcl-2 immunohistochemical expression can be found in Table 7 and Figure 9 below.

Table 13. Immunohistochemical Expression of Anti-Apoptotic Bcl-2

No	Rats Group	Intensity (I)	Areal (%)
1	K- U1	0	0
2	K- U2	0	0
3	K- U3	0	0
4	K- U4	0	0
5	K- U5	0	0
6	K- U6	0	0
7	K+ U1	+3	80
8	K+ U2	+3	75
9	K+ U3	+3	50
10	K+ U4	+3	75
1	K- U1	0	0
2	K- U2	0	0
3	K- U3	0	0
4	K- U4	0	0
5	K- U5	0	0
6	K- U6	0	0
7	K+ U1	+3	80
8	K+ U2	+3	75
9	K+ U3	+3	50
10	K+ U4	+3	75
11	K+ U5	+3	50
12	K+ U6	+3	50
13	P1 U1	+3	50
14	P1 U2	+3	50
15	P1 U3	+3	50
16	P1 U4	+3	70
17	P1 U5	+3	10
18	P1 U6	+3	20
19	P2 U1	+3	20
20	P2 U2	+3	20
21	P2 U3	+2	75
22	P2 U4	0	0
23	P2 U5	+3	50
24	P2 U6	+3	50
25	P3 U1	+3	50
26	P3 U2	+3	20
27	P3 U3	+3	20
28	P3 U4	+3	75
29	P3 U5	0	0
30	P3 U6	0	0

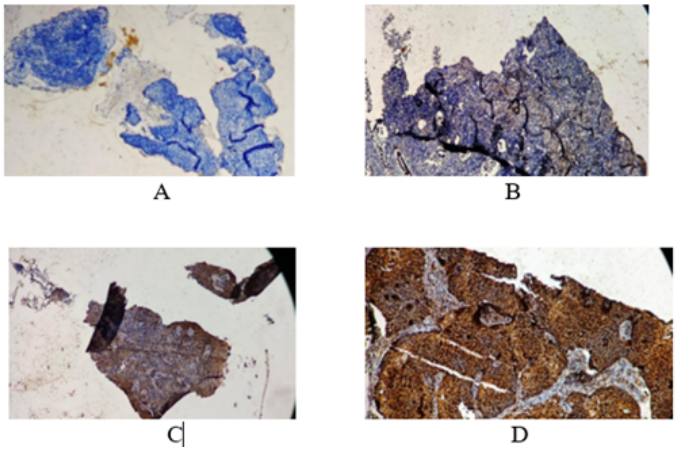


Fig. 18. Intensity of Bcl-2 Expression in Cervical Cancer. A. Score 0 (400x), B. Score 1 (100x), C. Score 2 (100x), D. Score 3 (400x)

4.12 Relationship Between Bcl-2 Expression Intensity in Cervical Cancer and Legundi Fruit (Vitex trifolia L.) Administration

Table 14. *Bcl-2 Expression (Allred score)* in Histopathological Types

Bcl-2 Expression (Allred score)	**Normal**		**Squamous Cell Carcinoma**		**Adenocarcinoma**	
	n	**%**	**n**	**%**	**n**	**%**
None-20%	11	36.7	5	16.7	0	0
50-75%	0	0	12	40	2	6.7
¿75%	0	0	1	3.3	0	0
Total	**11**	**36.7**	**18**	**60**	**1**	**3.3**
Total (%)			**30 (100)**		**p-value: 0.035**	

**Fisher's exact test: p¡0.05*

SPSS analysis using Fisher's exact test yielded a result of p=0.035 (p¡0.05), indicating a significant relationship between the loss or reduction of Bcl-2 expression in cervical cancer histopathology and the administration of legundi fruit extract.

Bcl-2 stabilizes cell membranes and prevents the release of apoptosis-inducing factors. When the Bcl-2/BAX complex forms, Bcl-2 loses its inhibitory activity. BAX migrates to the mitochondrial membrane upon receiving an apoptotic signal, triggering the release of cytochrome C and AIF into the cytosol. AIF enters the nucleus, causing chromatin condensation and nuclear fragmentation, followed by caspase activation. Bcl-2 plays a key role in radiation sensitivity. Increased

Bcl-2 expression is associated with poorer survival in cervical cancer patients, as Bcl-2-positive tumors show weaker responses to radiation. Bcl-2 expression levels after radiation therapy are useful prognostic markers. Chemotherapy resistance can occur through apoptosis inhibition, often due to mutations in the p53 gene. The ethanol extract of legundi fruit has shown potential in inhibiting tumor growth and p53 expression in skin cancer. Flavonoids like Casticin and Labdane-Type Diterpenes in legundi can inhibit cancer cell proliferation and induce apoptosis. This study suggests that legundi extract may be developed for future therapeutic use by targeting the Bcl-2 apoptosis pathway

5 Discussion

The extract of Vitex trifolia (legundi) contains compounds that exhibit anticancer properties by inhibiting cell proliferation, tumor growth, and p53 expression in epithelial cancer through the Bcl-2 apoptosis pathway. Flavonoids in legundi leaves, such as Casticin, have been shown to inhibit cancer cell proliferation in rats and activate apoptosis. Additionally, other studies report that rotundifuran, a Labdane-Type Diterpene found in legundi, can suppress cervical cancer cell proliferation in HeLa and SiHa cells. This research also highlights that Vitex trifolia contains Labdane-Type Diterpene, which has demonstrated superior interaction with Bcl-2 compared to Casticin. The study investigates the relationship between Bcl-2 expression intensity in cervical cancer and the administration of Vitex trifolia fruit extract. SPSS analysis using Fisher's exact test showed a significant result with p=0.035 (p¡0.05), indicating a relationship between the reduction or loss of Bcl-2 expression in the histopathology of cervical cancer and the administration of Vitex trifolia extract. This suggests that Vitex trifolia has potential as a therapeutic agent in reducing chemotherapy resistance in cervical cancer by modulating Bcl-2 expression.

6 Conclusion

The active compound Labdane-Type Diterpene found in Vitex trifolia extract exhibits stronger interactions with the Bcl-2 protein compared to Casticin and has shown the ability to suppress cervical cancer cell proliferation. This suggests that Labdane-Type Diterpene can be considered a potential cancer therapy candidate by inhibiting tumor growth through the Bcl-2 apoptosis pathway. In addition to molecular docking analysis, clinical analyses such as immunohistochemistry, histopathology, and kidney and liver function tests demonstrated a correlation between reduced Bcl-2 expression in cervical cancer histopathology and the administration of Vitex trifolia extract. This study confirms that ethanol extract of Vitex trifolia fruit is effective in inhibiting the Bcl-2 apoptosis pathway and holds promise for development as a future cancer therapy.

7 Acknowledgement

The authors would like to thank the Directorate General of Higher Education, Research, and Technology of the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia, as well as Universitas Muhammadiyah Sumatera Utara, for funding

References

1. F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA: A Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394–424, 2018.
2. N. Bhatla, D. Aoki, D. N. Sharma, and R. Sankaranarayanan, "Cancer of the cervix uteri: 2021 update," *International Journal of Gynecology & Obstetrics*, vol. 155, pp. 28–44, 2021.
3. L. V. Volkova, P. A. Ilexander, and N. O. Nadezhda, "Cervical carcinoma: Oncobiology and biomarkers," *International Journal of Molecular Sciences*, vol. 22, no. 22, pp. 1–22, 2021.
4. C. Gilham, A. Sargent, H. C. Kitchener, and J. Peto, "HPV testing compared with routine cytology in cervical screening: Long-term follow-up of ARTISTIC RCT," *Health Technology Assessment*, vol. 23, no. 28, pp. 1–43, 2019.
5. G. Gong *et al.*, "The Cyr61 is a potential target for rotundifuran, a natural labdane-type diterpene from *Vitex trifolia L.*, to trigger apoptosis of cervical cancer cells," *Oxidative Medicine and Cellular Longevity*, vol. 2021, 2021.
6. A. M. Almeida, J. A. Queiroz, F. Sousa, and Á. Sousa, "Cervical cancer and HPV infection: Ongoing therapeutic research to counteract the action of E6 and E7 oncoproteins," *Drug Discovery Today*, vol. 24, no. 10, pp. 2044–2057, 2019.
7. H. M. L. Lubis, "Kajian molekuler p53 pemanfaatan tanaman herbal buah legundi (*Vitex trifolia L.*) terhadap massa tumor jaringan kulit," *Buletin FARMATERA*, vol. 3, no. 1, pp. 41–48, 2018.
8. H. M. L. Lubis and I. Hariaji, "Ekstrak buah legundi (*Vitex trifolia*) mampu menghambat pembelahan dan pertumbuhan sel tumor kulit tikus," *Mutiara Medika: Jurnal Kedokteran dan Kesehatan*, vol. 17, no. 1, 2017. [Online]. Available: http://journal.umy.ac.id/index.php/mm/article/view/3676
9. E. W. C. Chan, S. K. Wong, and H. T. Chan, "Casticin from Vitex species: A short review on its anticancer and anti-inflammatory properties," *Journal of Integrative Medicine*, vol. 16, no. 3, pp. 147–152, 2018. [Online]. Available: https://doi.org/10.1016/j.joim.2018.03.001
10. Y. Nolvachai *et al.*, "Structure Elucidation Using Gas Chromatography-Infrared Spectroscopy/Mass Spectrometry Supported by Quantum Chemical IR Spectrum Simulations," *Analytical Chemistry*, vol. 93, no. 46, pp. 15508–15516, 2021. [Online]. Available: https://doi.org/10.1021/acs.analchem.1c03662
11. T. Lapidot-Cohen, L. Rosental, and Y. Brotman, "Liquid Chromatography–Mass Spectrometry (LC-MS)-Based Analysis for Lipophilic Compound Profiling in Plants," *Current Protocols in Plant Biology*, vol. 5, no. 2, pp. 20109, 2020. [Online]. Available: https://doi.org/10.1002/cppb.20109