Anticancer Activity Test Of 70% Alcohol Extract of Sembung Leaves (*Blumea Balsamifera*) Against Cervical Cancer Cells In-Vitro

Ni Luh Putu Dian Putri Permata Sari[1], *Ni* Luh Putu Eka Kartika Sari[2]*, *Putu* Nia Calista Santoso[2], *Erly* Sintya Dewi[2], *Desak* Putu Oki Lestari[3], *Asri* Lestarini[2], and *Ni* Wayan Armerinayanti[3]

[1]Undergraduate Student, Medicine Major, Faculty of Medicine and Health Sciences, Warmadewa University, Indonesia
[2]Department of Biochemistry and Physiology, Faculty of Medicine and Health Sciences, Warmadewa University, Indonesia.
[3]Department of Pathology and Anatomy, Faculty of Medicine and Health Sciences, Warmadewa University, Indonesia.

Abstract. Abstract. Cervical cancer is one of the most common cancers among Indonesian women, second only to breast cancer. Several anticancer medications cause minor to severe adverse effects such as fever, nausea, vomiting, hair loss, and other diseases. Because of the negative side effects suffered by patients, numerous researchs have been done to investigate herbal medicines as alternative cancer therapies with less side effects and more efficacy. The purpose of this study is to assess the efficacy of a 70% alcohol extract of *Blumea Balsamifera* (Sembung leaf) against cervical cancer cells. This study used a true experimental design with a quantitative methodology. The study used a 70% alcohol extract of *Blumea Balsamifera* leaves with concentrations of 15.625, 31.75, 62.5, 125, and 250 mg/ml, as well as HeLa cell cultures. Absorbance values were determined using the MTT assay and an ELISA Reader. Significant differences were discovered at concentrations of 125 and 250 mg/ml, while no significant differences were seen at concentrations of 15.625 and 31.25 mg/ml ($p > 0.05$). The study found that a concentration of 21.62 µg/ml of the extract reduces cell viability by up to 50%.
Keywords : Anticancer, Sembung, Hela, *Blumea Balsamifera*

1 Introduction

Cancer is one of the most prevalent causes of death, contributing to around 9.6 million deaths in various countries in 2018, according to Globocan data. Cancer is the seventh biggest cause of death in Indonesia, accounting for 5.7% of the total. The cancer incidence in 2008 was projected to be around 12.7 million cases. Following breast cancer, many women in Indonesia develop cervical cancer. The

* Corresponding author: kartikasari@warmadewa.ac.id

Ministry of Health reports that cervical cancer affects 23.4 per 100,000 people, with an average death rate of 13.9 per 100,000 (1). Some anticancer medications have more adverse effects than therapeutic benefits. Some of these include fever, nausea, vomiting, hair loss, and other symptoms. In addition to physical adverse effects, there are psychological side effects include rejection, anxiety, and loneliness (2). Currently, extensive study is being conducted on natural compounds that are thought to be able to treat. One source of natural compounds is the Usada Bali lontar, an ancient manuscript written by the ancestors of the Balinese community that contains information about treatments and diseases (3). The herbal plant known as sembung leaves (*Blumea Balsamifera*) is one of the candidates for herbal therapy mentioned in this lontar, recognized as a herbal rich in beneficial properties and containing several anticancer compounds. It includes a variety of chemical components, such as essential oils, flavonoids, and others.

The sembung leaf plant (*Blumea Balsamifera*) has been scientifically demonstrated to decrease the antiapoptotic Bcl-2 protein while raising the pro-apoptotic Bax protein. Furthermore, the sembung plant can improve chemotherapy therapy efficacy by blocking the P-gp efflux pump. This is owing to the accumulation of intracellular anticancer medications in cancer cells caused by the P-gp efflux pump, hence P-gp must be inhibited to raise intracellular anticancer drug concentrations (4).

Previous research has revealed the effects of sembung leaf extract (*Blumea Balsamifera*) on numerous cancer cells, such as NCI-H 187 lung cancer cells with an IC_{50} value of 1.29 µg/mL and T47D breast cancer cells with an IC_{50} value of 59.07 µg/mL. In addition to its notable effect on breast cancer cells, sembung leaves (*Blumea Balsamifera*) exhibit a significant anticancer effect, as evidenced by an IC_{50} value of 0.6342 mL/mL, indicating their potential as a natural therapeutic agent (4). Based on earlier findings, this study examined the effect of sembung leaf extract (*Blumea Balsamifera*) on HeLa cells.

2 Research Method

The research methodology is experimental using a quantitative approach. This work employs a real experimental design. The ingredients required are leaves from the sembung plant grown on the island of Bali. The hypothesis in this study was validated with the Statistical Package for the Social Sciences (SPSS) application. Post-hoc analysis was performed to assess the differences in significance across test groups. The IC_{50} value was calculated using Probit analysis. This research was submitted to the Health Research Ethics Commission (KEPK) of the Faculty of Medicine and Health Sciences, Warmadewa University, in compliance with regulation No.129/Unwar/FKIK/KEPK/2024, and was certified ethically feasible.

2.1 Extraction method

The samples, consisting of stems and leaves, were obtained in Tabanan, Bali, and were then desiccated in an oven at a temperature ranging from 50 to 60 degrees Celsius to achieve a stable dry mass. The extract was subsequently filtered through filter paper to obtain the filtrate, which was then concentrated in the dark using an evaporator, resulting in a crude extract. After 24 hours of the 48-hour maceration process, the mixture was filtered, and the sample was immersed in fresh 96% ethanol

2.2 Cultivation of Hela cervical cancer cells

Hela cells were grown in T25 flasks using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Germany), with 10% Fetal Bovine Serum (FBS) (Capricorn, Germany), 2% Penicillin-Streptomycin and 0.5% amphotericin B. The flasks were incubated at 37°C with 5% CO2.

The cytotoxic potential of *Ocimum sanctum* was evaluated using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Hela cells were grown to 80% confluence in a 96-well plate and incubated at 37°C with 5% CO2 for 24 hours. The cells were washed with Dulbecco's Phosphate Buffer Saline (DPBS) (Capricorn, Germany) before treatment. They were divided into three groups: untreated (NT), control medium without cells, and various concentrations of eugenol (15.625, 31.25, 62.5, 125, and 250 µg/ml). The treated cells were incubated at 37°C with 5% CO2 for another 24 hours. After removing the media and washing with DPBS, 0.5 mg/ml MTT reagent was added at 100 µl per well. The cells were incubated for four hours, then treated with 100 µl of Dimethyl sulfoxide (Sigma-Aldrich, Germany) to stop formazan crystal production (Wihadmadyatami *et al*., 2023). The samples were measured at 590 nm using an ELISA reader, and cell viability was calculated as follows:
Viability = ((Absorption of treated samples x 100) / Absorption of controls)

3 Result and Discussion

The proper solvent must be chosen carefully in order to obtain the desired chemical product. Solvents such as methanol, 70% alcohol, methanol, ethyl acetate, and N-hexane are commonly utilized. In this investigation, 70% alcohol was utilized as a solvent because it is considered safe for consumer usage. According to Quintana *et al*., 70% alcohol can boost the sensitivity of cancer cells to specific physical and chemical anticancer agents. Acetaldehyde will be produced by all processes associated with the metabolism of 70% alcohol. Acetaldehyde production can contribute to oxidative stress by producing reactive oxygen species. 70% alcohol can also stimulate genistein-induced apoptosis in cervical cancer cells (5).

According to the proportion of alcohol extract that is suitable for consumption, 70% is considered safe. There were no acute or subacute abnormalities in the 96% alcohol extract of sembung leaves (*Blumea Balsamifera*) in the study by Nhung & Quoc (2020), nevertheless mice did experience side effects from the extract (6). The maceration approach is used in this study because the extract does not need to go through a high-temperature process, which might harm components that are not resistant to high temperatures. Badaring *et al*. refer to the maceration approach as cold extraction since it does not need heating the sample or solvent to make the chemicals thermolabile (7). Heating-based extraction can degrade flavonoid and phenolic compounds. Gas Chromatography Mass Spectrophotometry (GCMS) is one of the technologies available for determining secondary metabolites in testing material. Table 1 presents the results of screening sembung leaves with GCMS and table 2 shows the content in sembung leaves that have the function of inhibiting the life of cancer cells.

Table 1. Phytochemical results of sembung leaves using the GCMS method

Compound Name	% Area	Qual (highest to lowest)

Propenamide, 2-cyano-3-(2,4,6-trimethylphenyl)	0.06	98
Propenamide, 2-cyano-3-(2,4,6-trimethylphenyl)	0.48	96
o-Veratramide	0.40	96
Bicyclo[2.2.1]heptan-2-one, 1,7,7- trimethyl-, (1S)-	14.44	96
1-Naphthalen-1-yl-2-(2-trifluoromethyl-imidazolidin-2-yl)-ethanone	0.66	95
Copper, bis (4-chloro-3,5-cyclohexadiene-1,2-dione 2-oximato-N2,O1)	2.59	90
Neophytadiene	5.10	83
9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3.beta.,5Z,7E)	0.73	80
Alanyl-.beta.-alanine	0.25	76
Propanamide, 3-(3,4-dimethylphenylsulfonyl)	0.61	76
Propanamide, 3-(3,4-dimethylphenylsulfonyl)	0.50	76
Piperidine-3-carboxylic acid, 1-ethylthiocarbamoyl-, amid	0.36	76
S-[Tri-t-butoxysilyl]-2-mercaptoethylamine	0.28	76
2-Iodohiistidine	1.63	76
Alanyl-.beta.-alanine	0.72	76
1,2,5-Oxadiazol-3-carboxamide, 4,4 '-azobis-, 2,2'-dioxide	6.63	76
(Cyclohex-3-enylmethyl)[2-(2-methyl-5-trifluoromethoxy-1H-indol-3-yl)ethyl]amine	1.48	76
Pregn-5-ene-3,20-diamine, (3.beta.,20S)-	1.93	76
p-Benzenediacetohydroxamic acid 1-Octadecanamine, N-methyl-	0.98	76
Desmethyldoxepin	1.28	68
Propanamide, 3-(3,4-dimethylphenylsulfonyl)	0.58	68
Propanamide, 3-(3,4-dimethylphenylsulfonyl)	1.01	68
3-Methyl-3,5--(cyanoethyl)tetrahydro-4-thiopyranone	1.09	68
Benzo[h]quinoline, 2,4-dimethyl-(E)-2-bromobutyloxychalcone	6.31	68
Benzo[h]quinoline, 2,4-dimethyl	0.03	68
Benzo[h]quinoline, 2,4-dimethyl	0.78	68
Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)	1.61	66
Benzaldehyde, 2-nitro-, diaminomethylidenhydrazone	0.47	64

Table 2. Biological function of compounds

Class of compounds	Compound Name	Function
Flavonoid	Benzo[h]quinoline, 2,4-dimethyl-(E)-2-bromobutyloxychalcone	Anticancer, Antioxidant, Antimalarial, Antiviral, Antibacterial, Antidiabetic, Antifungal (8).
Alkaloid	1-Naphthalen-1-yl-2-(2-trifluoromethyl-imidazolidin-2-yl)-ethanone	Antitumor, anti-inflammatory, anticonvulsant, analgesic, antiarrhythmic, diuretic (8).
	(Cyclohex-3-enylmethyl)[2-(2-methyl-5-trifluoromethoxy-1H-indol-3-yl)ethyl]amine	
	o-Veratramide	
	Piperidine-3-carboxylic acid, 1-ethylthiocarbamoyl-, amid	
Terpenoid	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	Anticancer, Anti-inflammatory, Antiviral,

	Neophytadiene	Antibacterial, Antimalarial (9)
	3-Methyl-3,5--(cyanoethyl)tetrahydro-4-thiopyranone	
	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	
Steroid	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3.beta.,5Z,7E)-	Anticancer, Antitumor, Antibacterial, Anti-inflammatory (9).
	Pregn-5-ene-3,20-diamine, (3.beta.,20S)-	
Metal complex	Copper, bis(4-chloro-3,5-cyclohexadiene-1,2-dione 2-oximato-N2,O1)-	Antitumor, Antimicrobial, Anti-inflammatory (9).
Amida	Propanamide, 3-(3,4-dimethylphenylsulfonyl)-	Anticancer, Anti-inflammatory, Antibacterial, Antiviral (10).
	Propenamide, 2-cyano-3-(2,4,6-trimethylphenyl)-	
	Piperidine-3-carboxylic acid, 1-ethylthiocarbamoyl-, amid	
Organosilicon	S-[Tri-t-butoxysilyl]-2-mercaptoethylamine	Anticancer (10)
Amino acids	2-Iodohyistidine	Antioxidant, Antitumor, Anti-inflammatory (10)
	Alanyl-.beta.-alanine	
Heterocyclic (oxadiazole)	1,2,5-Oxadiazol-3-carboxamide, 4,4'-azobis-, 2,2'-dioxide	Antioxidant, Anticancer, Anti-inflammatory, Antibacterial, Antiviral, Antifungal (11)
Aromatic	Desmethyldoxepin	Antioxidant, Antitumor, Anticancer, Anti-inflammatory, Antimicrobial (11)
	Benzaldehyde, 2-nitro-, diaminomethylidenhydrazone	
	p-Benzenediacetohydroxamic acid 1-Octadecanamine, N-methyl-	

Table 3 displays the results of the MTT test at a wavelength of 595 NM used in this study. Picture 1 depicts the morphology of death live cell hela in a wellplate with various concentrations, while MTT result showed at Picture 3 that displays the anticancer test findings for a 70% alcohol extract of sembung leaves (*Blumea Balsamifera*), which has an IC_{50} of 21.62 µg/ml.

Table 3. MTT Test Results with Wavelength 595 NM

Concentration of Sembung extracts (µg/ml)	Average Absorbance	Cell viability	Standard Deviation
0	0.98	100%	0.985±0.98
15,625	0.84	76.46%	0.839±0.84
31.25	0.53	26.74%	0.533±0.53
62.5	0.50	21.61%	0.501±0.50
125	0.41	6.86%	0.410±0.41
250	0.42	8.97%	0.423±0.42

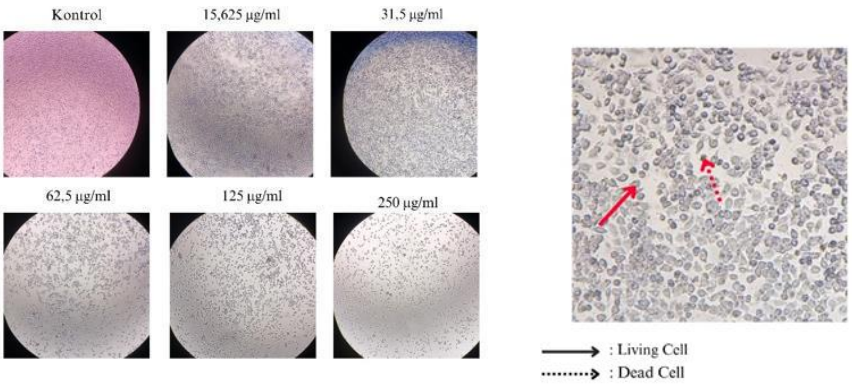

Fig 1. Test Results of Sembung Leaf Extract on HeLa Cells.

The Saphiro-Wilk test revealed that only the group receiving extract at a dose of 125 µg/ml exhibited an abnormal distribution ($p < 0.05$). The normality test results can be found in Table 4.

Table 4. Normality Test Results.

Treatment Group	Significance (p-value)
Control	1,000
15,625	0.611
31.25	0.286
62.5	0.798
125	0,000*
250	0.165

A test was performed to investigate the relevance of the influence of 70% alcohol extract of sembung leaves (*Blumea Balsamifera*) on MTT test results. The Kruskal Wallis test was used to conduct the test on non-normally distributed data. The Kruskal-Wallis test revealed a significant difference in mean MTT between groups ($p = 0.008$). A post-hoc test was then used to establish the significance of the differences between groups in greater detail (Table 5). The post-hoc test found no significant difference between the control group and concentration groups (15.625 and 31.25 µg/dl).

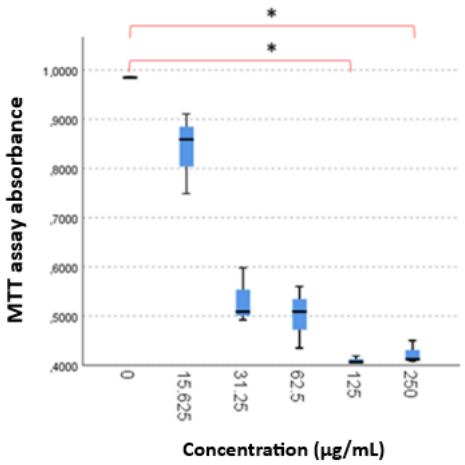

Fig 2. Histogram Graph of MTT Differences Between Control and Test Groups. Asterisks indicate significant differences ($p < 0.05$)

Table 5. Post-hoc Test Table. Asterisks indicate significant differences ($p<0.05$)

Group Comparison	*Test Statistics*	*Std. Error*	*Std. Test Statistic*	Sig.
125-250	-2,000	4,354	-0.459	0.646
125-62.5	-6,167	4,354	-1,416	0.157
125-31.25	-7,167	4,354	-1,646	0.100
125-15.625	-11,333	4,354	-2,603	0.009*
125-0	14,333	4,354	3,292	0.001*
250-62.5	-4,167	4,354	-0.957	0.339
250-31.25	-5,167	4,354	-1,187	0.235
250-15,625	9,333	4,354	2,143	0.032*
250-0	12,333	4,354	2,832	0.005*
62.5-31.25	1,000	4,354	0.230	0.818
62.5-15.625	5,167	4,354	1,187	0.235
62.5-0	8,167	4,354	1,875	0.061
31.25-15.625	4,167	4,354	0.957	0.339
31.25-0	7,167	4,354	1,646	0.100
15.625-0	3,000	4,354	0.689	0.491

IC_{50} study revealed that a concentration of 21.63 µg/ml of extract was required to reduce cell viability to 50% IC_{50}= 21.62 µg/ml).

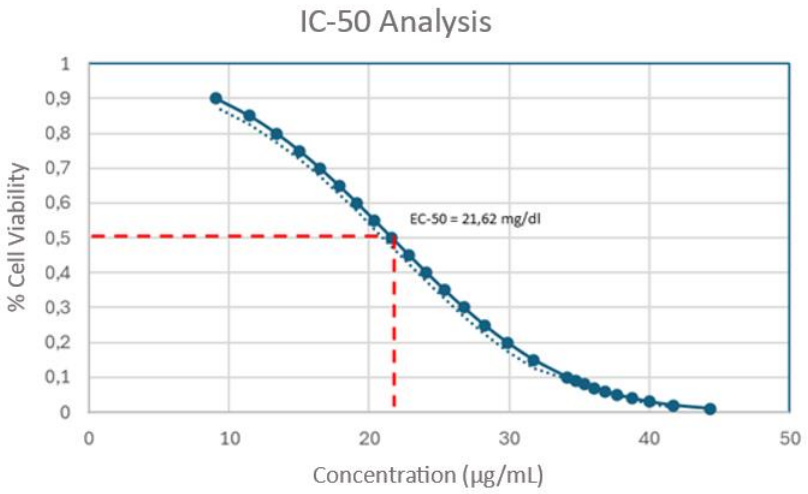

Fig 3. IC-50 Analysis Graph of the Relationship between Concentration and Percentage of Cell Viability.

According to Amaliah *et al*., chemical activity is divided into several classes depending on the IC_{50}. A compound has a strong anticancer if the IC_{50} value is less than 50 µg/mL, a strong anticancer if the IC_{50} is 50-100 µg/mL, a moderate anticancer if the IC_{50} value is 101-150 µg/mL, and a weak anticancer if the IC_{50} value is 151-200 µg/mL (12). The IC_{50} in this investigation was 21.62 µg/mL, indicating a high level of anticancer activity. The IC_{50} in this investigation was 21.62 µg/mL, indicating a high level of anticancer activity. The results of this phytochemical screening show that the 70% alcohol extract of sembung leaves (*Blumea Balsamifera*) contains a variety of phytochemical substances, including alkaloids, terpenoids, steroids, and flavonoids. Figure 4 depicts the mechanism of many secondary chemicals used as anticancer agents.

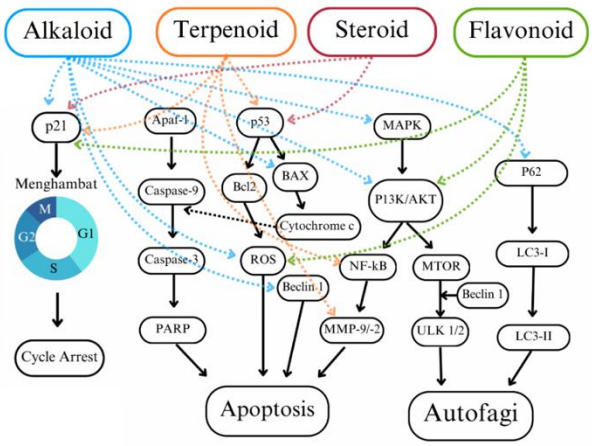

Fig 4. Mechanism of Secondary Compounds as Anticancer.

Alkaloid substances can be classified using a variety of criteria. This study classified alkaloids based on their chemical structure, and numerous categories were established, including isoquinoline, indole, and piperidine. Previous study reported that isoquinolone alkaloids can cause cell death through apoptosis, autophagy, and cell cycle arrest. According to Luo *et a (2022)*., indole alkaloids can activate autophagy via targeting the PI3K/Akt/mTOR signaling pathway, the MAPK signaling route, Beclin-1, the ROS signaling pathway, and P62/SQSTM1. Mitra *et al*. found that pepiridine alkaloids can act as anticancer agents by inhibiting cancer cell growth and differentiation via a variety of pathways, including ROS generation, intrinsic and extrinsic caspase-mediated apoptosis pathways, inhibition of cancer cell migration, suppression of oncogene expression, increased cytochrome c synthesis, and mitochondrial-mediated BAX-2 (13).

Terpenoid compounds are classified as monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids. In the study of Kamran *et al*., stated that Monoterpenoids can also be anticancer agents by inhibiting growth, suppressing cell migration and metastasis, reducing the expression of matrix metalloproteinases (MMP-2 and MMP-9), and targeting the p53 signaling pathway in HeLa cells. Sesquiterpenoids can also be anticancer agents in HeLa cells by targeting ROS generation, sarcoplasmic reticulum calcium ATPase pumps, NF-KB, p53 signaling pathways, angiogenesis and metastasis. Diterpenoids can induce p21 expression by suppressing CDKs through the p53 mechanism and stopping the G1 phase, and suppressing the function of DNA and RNA polymerase II followed by inhibition of proliferation by blocking EGFR, p-Cdc-2, p-Cdc25c, cyclin B1 and inducing apoptosis by changing the expression levels of Bax and Bcl-2 in HeLa cells. Triterpenoids can arrest the S phase of the cell cycle and can be mediated by mitochondria by stimulating superoxide generation, and can suppress the proliferation of HeLa cells mediated by upregulation of caspase 3 and initiation of apoptosis (14).

Steroids can induce apoptosis and inhibit the cell cycle in cancer. This is stated in the study of Sirait *et al*., that steroids can damage the permeability of the mitochondrial membrane so that it can activate the apoptosis process. In addition to inducing apoptosis, steroids can stop the cell cycle. This was revealed in the study of Bafadal *et al*., steroids can inhibit cancer cell proliferation by targeting the G1 cell cycle phase so that it does not continue to the S phase in the cell cycle.

According to Luna *et al*., flavonoids mediate anti-neoplastic mechanisms by modulating reactive oxygen species (ROS) levels in tumor cells, inhibiting carcinogens, pro-inflammatory pathways, angiogenesis, autophagy, inducing apoptosis, and inhibiting tumor proliferation and invasion. In the study by Kopustinskiene *et al*., flavonoids as anticancer agents work by modulating ROS-scavenging activity, inhibiting the cell cycle, inducing apoptosis by targeting apoptosis cascade signaling, autophagy, and suppressing cancer cell proliferation and invasion (15).

4 Conclusion

The study found that 70% alcohol extract of sembung leaves (*Blumea Balsamifera*) had anticancer action against HeLa cervical cancer cells, with an IC_{50} cytotoxic test value of 21.62 µg/ml, making it a potent anticancer.

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