Cytotoxicity of Soursop (*Annona Muricata* L.) Seed Protein on HeLa and Vero Cells

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Abstract. Cervical cancer was the fourth-highest incidence of female cancer globally in 2020 and the second-highest incidence of female cancer in Indonesia in 2019. Chemotherapy frequently causes side effects and resistance; thus, cancer therapeutic agents must be developed to find selective anticancer drugs. One of the plants that has the potential as an anticancer agent is soursop. Soursop seed extract demonstrated anticancer activity, but research on soursop seed protein has never been published. This research aims to determine the cytotoxicity of soursop seed protein (*Annona muricata* L.) isolated using DEAE matrix against HeLa and Vero cells. Soursop seeds were extracted by adding 0.14 M NaCl at 4 °C in 5 mM sodium phosphate buffer pH 7.2. Protein purification was done by ion-exchange column chromatography with stationary phase DEAE matrix and mobile phase NaCl ranging from the lowest molarity of 0.2 M to the highest molarity of 0.4 M. Protein fraction concentrations were measured using Biodrop at A260/280. MTT assay was employed to test the cytotoxic activity of protein fraction soursop seed, followed by measurement of cells' metabolic activity with ELISA reader at λ 550 nm. The results of protein isolation showed that the highest concentration of protein fraction eluted 0.2 M NaCl were 8466.2 μg/mL; 8346.4 μg/mL; 8205.0 μg/mL; and 8284.0 μg/mL. Cytotoxic tests using soursop seed protein with the concentrations of 32.2 μg/mL; 16.1 μg/mL; 8.05 μg/mL; and 4.025 μg/mL respectively resulted in 114.436%; 116.822%; 117.776%; and 119.526% HeLa cell survival. Meanwhile, isolated proteins with the concentration of 23.7 g/mL; 11.85 g/mL; 5.925 g/mL; and 2.9625 g/mL respectively resulted in 90.671%; 115.284%; 123.025%; and 127.590% of Vero cell survival. In conclusion, soursop seed protein did not possess cytotoxic activity against HeLa and Vero cells.

Keywords: Soursop seed · DEAE · MTT assay · HeLa cell · Vero cell

1 Introduction

Cancer is a disease that causes abnormal and uncontrolled cell growth, allowing it to spread to all parts of the body [1]. Cancer is one of the world's leading causes of death. Statistical data from the Global Burden of Cancer (GLOBOCAN) released by

A. Sri Wahyuni et al. (Eds.): ICB-Pharma 2022, AHCPS 3, pp. 329–338, 2023.
https://doi.org/10.2991/978-94-6463-050-3_28

the International Agency for Research on Cancer (IARC) reported 19.3 million cancer cases and 10 million cancer deaths in 2020. The global cancer incidence is estimated to increase up to 30.2 million cases by 2040. Cervical cancer was the world's fourth most common cancer in women with 604,127 cases [2]. In Indonesia, cervical cancer was the second-highest incidence of female cancer after breast cancer, with 23.4 cases per 100,000 population and an average death rate of 13.9 per 100,000 population [3]. The main cancer treatment modalities in Indonesia are chemotherapy, radiotherapy, and surgery [1]. Nonetheless, their long-term use often results in side effects and resistance because chemotherapeutic agents lack selectivity against cancer cells. [4]. One of the strategies to resolve this problem is the development of new drugs as selective anticancer drugs.

Advances in science and technology have resulted in significant changes in our understanding of molecular biology and biochemistry over the last few decades. Discoveries of the role of protein in the treatment of disease offer several advantages over other conventional treatments, including the properties of proteins that can provide very specific functions to minimize the occurrence of side effects and disturbances in normal body biological functions [5]. An example of the application of protein's role in disease treatment in clinical practice is cancer therapy using monoclonal antibodies (mAbs) because of their ability to attack the tyrosine kinase receptor specifically so that there is no stimulation of cell proliferation [6].

New anticancer agents can be developed by utilizing RIPs (Ribosome Inactivating Proteins) in a plant. RIPs are toxic proteins that are widely distributed in higher plants, as well as fungi and bacteria [7]. The presence of RIPs in a plant can be identified through their enzymatic activity in cutting double-stranded supercoiled DNA into nick circular DNA or linear DNA in vitro [8]. The potent cytotoxic activity of RIPs can be used as candidates for the development of immunotoxins for cancer therapy [9].

Soursop (*Annona muricata* L.) is a plant in the genus *Annona*. There has been no additional research into the protein activity of soursop seed. Sugar apple seeds (*Annona squamosa*), a plant in the same genus as soursop, indicated the presence of RIP-like protein content through its activity in cutting double-stranded supercoiled pUC19 DNA into nicked circular DNA, with a total protein content of 25 μg [10]. With an IC_{50} value of 43.02 μg/mL, RIP from sugar apple seeds had cytotoxic activity against gastric cancer cells[11].

RIPs or plant proteins can be isolated using ion-exchange column chromatography with DEAE matrix to obtain proteins, which are then tested for their cytotoxicity activity on HeLa and Vero cells. This study aims to test the cytotoxicity of soursop seed protein (*Annona muricata* L.) isolated using the DEAE matrix against HeLa and Vero cells. The results are expected to provide scientific data on the cytotoxic activity of soursop seed protein (*Annona muricata* L.) isolated using the DEAE matrix against HeLa and Vero cells.

2 Methods

2.1 Tools

The tools used in this research were glassware, autoclave, oven, centrifuge (Mikro 200 R Hettich Zentrifugen), micropipette (Socorex), analytical balance (Ohaus), pH meter (Electrofac Merrohm), Biodrop (Biodrop DUO), yellow tip, white tip, blue tip, Eppendorf (Biorad), conical tube, refrigerator, CO_2 incubator (Binder), ELISA reader (Biotek Elx800), cytotoxic safety cabinet (ESCO), camera (Opti lab), 96 well plates (Nunc), hemocytometer, and inverted microscope (Zeiss).

2.2 Materials

The materials used were soursop seeds obtained from Pasar Kleco Surakarta, sodium phosphate buffer, NaCl, NaOH 2M, aquades, HCl 2M, DEAE-650M matrix, aluminum foil, HeLa cervical cancer cells from the Mammal Cell Culture Laboratory of Pharmaceutical Biology Section Universitas Muhammadiyah Surakarta, Vero cells from the stock of BPTO Karanganyar Laboratory, Phosphate Buffered Saline (PBS), trypsin-EDTA, MTT reagent (3-(4,5- Dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide), stopper reagent in the form of Sodium Dodecyl Sulphate (SDS) 10% in 0.01 N HCl, doxorubicin, Dulbecco's Modified Eagle Medium (DMEM) culture medium, Roswell Park Memorial Institute culture medium 1640 (RPMI 1640), Fetal bovine serum (Gibco) 10% v/v, penicillin-streptomycin (Gibco 1% v/v), fungison (Gibco) 0.5% (v/v), sodium bicarbonate, and hepes.

2.3 Soursop Seed Protein Extraction

Soursop seeds were washed with running water, weighed 50 g, cut into small pieces, crushed with a cold mortar, and extracted with 50 mL of 0.14 M NaCl solution at 4 °C in 5 mM sodium phosphate buffer pH 7.2. The resulting extract was squeezed using a small screen printing filter. The liquid obtained was centrifuged cold at a speed of 7,000 rpm for 5 min. The obtained supernatant was sapwood extract and it was stored at 4 °C [12].

2.4 Soursop Seed Protein Isolation

The supernatant from soursop seeds was isolated using the DEAE matrix. The DEAE matrix was placed in a column in the form of a 10 mL injection syringe and eluted using 5 x volume of sterile distilled water, or approximately 50 mL. The DEAE matrix was then eluted with 10 mL of 5 mM sodium phosphate buffer pH 7.2. 10 mL sapwood extract sample in the form of supernatant was placed in the column and eluted, then, the eluent was accommodated in a test tube. Following that, the eluent of 10 mL 0.2 M NaCl solution and 10 mL 0.4 M NaCl ranging from the lowest molarity were eluted into the column, and the resulting eluents were also accommodated in a marked test tube. Each of the protein fractions was measured for protein concentration. Protein concentration was determined by taking 2 μL of sapwood extract and isolated protein fractions and measuring them using Biodrop at a wavelength of 260/280 nm with a blank of 5 mM sodium phosphate buffer pH 7.2. The results of the highest protein fraction concentration were selected as the samples for cytotoxic tests [13].

2.5 MTT Cytotoxic Assay

After 80% of the cells were confluent, cell harvesting could be carried out. The RPMI and DMEM media that had been used to grow HeLa and Vero cells were discarded. After neutralizing the cells with 5 mL of PBS, they were homogenized and the PBS was removed. Then, 450 μL of trypsin-EDTA was poured into the cells, and the cells were incubated in a 5% CO_2 incubator at 37 °C for 5 min to prevent cells from clumping together. 5 mL of media from each cell was added to the cells, which were then resuspended. The cells were moved into a sterile conical tube. 10 μL of cell suspension in a conical tube was taken and counted with a hemocytometer. 100 μL of each suspension HeLa and Vero cells were put into every 96 well plates at a density of 10,000 cells per well and incubated for 24 h for adaptation.

The highest soursop seed protein concentration was used as the sample for the MTT assay. Following the plate layout, for each of 96 well plates HeLa and Vero cells, samples of soursop seed protein fraction were treated using four concentration series; positive control (culture media + cells + doxorubicin); cells control (cells + culture medium); media control (media only); and doxorubicin control (culture media + doxorubicin). The cells were then incubated for 24 h at a 5% CO_2 incubator at 37 °C. After 24 h, 100 μL of MTT reagent was added to every 96 well plates and incubated for 4 h in a 5% CO_2 incubator at 37 °C until formazan crystals formed. Then, 100 μL of stopper reagent was put in every 96 well plates. Furthermore, the 96 well plates were coated with aluminum foil and stored at room temperature away from direct light. The absorbance of survived cells was measured the next day using a 550 nm ELISA reader [14].

Data absorbance of survived cells was calculated to get the percentage of survived cells using the following formula:

$$\frac{(\text{Abs. treated groups} - \text{Abs. media control})}{\text{Abs. cells control} - \text{Abs. media control}} \times 100\% \quad (1)$$

A graph of concentration vs % survived cells was created to obtain the linear regression equation $Y = bX + a$. The value of 50% was substituted for Y to obtain the X value. IC_{50} was the antilog of the X value.

3 Results and Discussion

3.1 Soursop Seed Protein Extraction

The extraction process aims to produce a sapwood extract of soursop seed protein. Protein extraction was performed by adding salt in the form of 0.14 M NaCl at 4 °C in 5 mM sodium phosphate buffer pH 7.2. Extraction takes place at a low temperature (4 °C) to avoid denaturation of the protein when it is released from the cell [15]. The use of low concentration NaCl (<0.5 M) causes salting in, which increases protein solubility [16]. As a result, the protein dissolved in the sodium phosphate buffer will separate more easily during filtration. From the extraction, 36 mL of sapwood extract was obtained.

3.2 Soursop Seed Protein Isolation

Soursop seed sapwood extract was purified using the ion-exchange chromatography technique with the stationary phase DEAE-650M matrix and the mobile phase (eluent) NaCl. Ion exchange chromatography is a protein purification technique that uses electrostatic interactions between positively and negatively charged protein amino acid groups and their stationary phase (matrix) [17]. Diethyl-aminoethyl-cellulose (DEAE) is a weakly positively charged anion exchange matrix [18]. The DEAE matrix can work in the pH range of 2–9 [19]. The use of 5 mM sodium phosphate buffer pH 7.2 aims to maintain and avoid pH fluctuations that can occur when the matrix and protein are eluted by the mobile phase [19]. NaCl was chosen as the mobile phase because it does not affect the protein structure and it is the most commonly used for protein separation [19]. The DEAE matrix can function at a salt concentration ranging from 0.1 to 1.6 M [15]. In this study, NaCl eluent was used with an increasing concentration gradient from 0.2 M NaCl to 0.4 M NaCl.

When the DEAE matrix and a weak ionic strength buffer solution reach equilibrium, the positively charged DEAE matrix adsorbs the negatively charged proteins, causing the positively charged proteins to elute earlier [20]. Then, the negatively charged protein that binds to Na^+ ions of NaCl will be eluted into the test tube [20]. The ionic strength increases as the eluent concentration gradient increases [19]. The position of the negatively charged protein will be replaced by the eluent with a higher ionic strength [19].

Protein concentration was measured using Biodrop at a wavelength of 260/280 nm. The Biodrop principle states that DNA containing purine and pyrimidine bases can absorb UV light at a wavelength of 260 nm, while at a wavelength of 280 nm, it becomes the maximum absorption region of the protein [21]. An absorbance ratio of 260/280 ~ 1.8 indicates the presence of pure DNA, an absorbance ratio of 260/280 ~ 2.0 indicates the presence of pure RNA, and the lower ratio of 1.8–2.0 indicates the presence of protein; phenol; or other contaminants that strongly absorb at a wavelength of 280 nm [22]. The estimated protein concentration with an optical density ratio of 280 to an optical density of 260 of 1.75 indicates the presence of 0.00% nucleic acid [23]. From the isolation of soursop seed protein with DEAE-650 matrix measured using Biodrop at cold temperatures, the highest protein concentration was found in the soursop seed protein fraction with 0.2 M NaCl mobile phase, which was 8466.2 μg/mL; 8346.4 μg/mL; 8205.0 μg/mL; and 8284.0 μg/mL with an A260/A280 ratio respectively of 1.160; 1.176; 1.015; and 1.015 as shown in Table 1. Based on the A260/A280 ratio data from the soursop seed protein fraction, it can be concluded that the soursop seed protein fraction has low purity, indicating nucleic acid contamination still exists. Protein extraction and isolation can be optimized to obtain a higher purity protein. Following the extraction of the sample, the protein is separated from other contaminants using the precipitation method with ammonium sulfate, followed by dialysis, and then purified using ion-exchange chromatography techniques [13].

3.3 MTT Cytotoxic Assay

The cytotoxic test assesses the potential toxicity of natural materials to a cell [24]. This study used the MTT cytotoxic test method in vitro. The MTT method is based on the

Table 1. Protein concentration isolated using DEAE-650M matrix

Sample	Test tube to-(5 mL per tube)	Protein Concentration (μg/mL)				Ratio A260/A280			
		*	**	***	****	*	**	***	****
Sapwood extract of soursop seed	1	35.20	50.40	32.40	98.4	1.750	1.625	1.333	1.500
	2	4665.0	4541.0	559.8	522.8	1.101	1.115	1.660	1.640
NaCl 0,2 M	1	8466.2	8346.4	8205.0	8284.0	1.160	1.176	1.015	1.015
	2	3065.6	3034.6	4784.4	4752.2	1.358	1.363	0.983	0.980
NaCl 0,4 M	1	1454.8	1439.0	1278.4	1261.4	1.476	1.479	1.231	1.218
	2	690.0	689.4	893.0	860.8	1.512	1.506	1.421	1.424

*First reading of soursop seed protein fraction concentration for cytotoxic assay on HeLa cells
** Second reading of soursop seed protein fraction concentration for cytotoxic assay on HeLa cells
*** First reading of soursop seed protein fraction concentration for cytotoxic assay on Vero cells
**** Second reading of soursop seed protein fraction concentration for cytotoxic assay on Vero cells

ability of the succinate dehydrogenase enzyme, which is found in the mitochondria of living cells, to enzymatically reduce the yellow MTT tetrazolium salt to purple formazan crystals [25].

Formazan crystals are insoluble in water but dissolve in 10% SDS. SDS 10% can denature cell proteins into polypeptide units. The absorbance value of formazan crystals can be read using an ELISA reader at 550–600 nm. The greater intensity of the purple color indicates that the number of survived cells is increasing [26].

The success of the MTT cytotoxic test was measured by the IC_{50} value. IC_{50} value denotes concentrations that can cause death in 50% of the cell population [24]. The smaller the IC_{50} value, the greater the cytotoxic potential [26]. According to the National Cancer Institute and the Geran Protocol criteria, compounds with an IC_{50} value of 20 μg/mL are classified as a highly toxic effect; IC_{50} values between 21 and 200 μg/mL are classified as moderate toxic effects; IC_{50} values in the range of 201–500 μg/mL are classified as a weak toxic effect, and IC_{50} values > 501 μg/mL are classified as non-toxic [27].

This study used HeLa and Vero cell lines. HeLa cells are cervical cancer cells that undergo rapid division when the human papillomavirus (HPV) type 18 E7 gene in these cells is suppressed by the bovine papillomavirus (BPV) E2 protein [28]. While Vero cells are normal cells (non-tumorigenic) that are long or oval, have many microvilli, heterochromatin forms clumps and large amounts of euchromatin are evenly distributed in the nucleus, mitochondria are round or long with cristae located near the nucleus, and free ribosomes are observed in the cytoplasm or bound to the endoplasmic reticulum [29].

Doxorubicin is an anthracycline antibiotic that is widely used as cancer therapy. It has various mechanisms of action including inhibition topoisomerase II and intercalation DNA [30]. As shown in Table 2, the IC_{50} results of doxorubicin as positive control on HeLa cells were 5.60 μg/mL. Meanwhile, doxorubicin had IC_{50} values of 8.70 μg/mL in Vero

Table 2. Data of soursop seed protein cytotoxic test isolated using DEAE-650M (NaCl 0.2 M) against HeLa cells at λ 550 nm

Sample	Concentration (μg/mL)	Average of % Survival Cells	IC50 (μg/mL)
Fraction of NaCl 0.2 M	32.2 16.1 8.05 4.025	≥100 ≥100 ≥100 ≥100	–
Doxorubicin	50 25 12.5 6.25 3.125	28.378 39.692 45.797 45.956 55.341	5.60

Table 3. Data of soursop seed protein cytotoxic test isolated using DEAE-650M (NaCl 0.2 M) against Vero cells at λ 550 nm

Sample	Concentration (μg/mL)*	Average of % Survival Cells	IC50 (μg/mL)
Fraction of NaCl 0.2 M	23.7 11.85 5.925 2.9625	90.671 ≥100 ≥100 ≥100	–
Doxorubicin	25 12.5 6.25 3.125	28.345 48.293 53.355 68.837	8.70

cells (Table 3). Doxorubicin has been tested for its ability to kill cancer cells and is more sensitive to HeLa cancer cells. It has an IC_{50} value which is classified as very toxic [31]. The existence of resistance causes doxorubicin to be toxic to normal cells [30].

In this study, the results of the cytotoxic test of soursop seed protein fraction samples against HeLa and Vero cells were obtained from absorbance readings using ELISA reader 550 nm (Table 2 and Table 3). The results revealed that an increase in protein concentration resulted in a lower percentage of survived cells (dose-dependent). Soursop seed protein did not have cytotoxic activity against HeLa and Vero cell lines. Of the 4-concentration series used, all protein fractions of soursop seeds produced the percentage value of survived cells more than 50% so IC_{50} could not be calculated. Meanwhile, the results of research from Dewi and Indrayudha (2021, unpublished data) showed that the protein fraction of soursop seed was moderate toxic to 4T1 cells, with an IC_{50} value of 73.96 ± 25.10 μg/mL and had no antiproliferative activity. Another study conducted by Rahma and Indrayudha (2021, unpublished data) reported that the protein fraction of soursop seed had weak toxicity to MCF-7 cells, with an IC_{50} value of 429.90 ±

9.77 μg/mL and had no antiproliferative activity. One of the factors influencing the difference in results of cytotoxic tests in vitro is the use of soursop seeds obtained from Pasar Kleco Surakarta, which may not have come from the same source. It is suggested that future researchers obtain the soursop seeds from the same plant source to produce the same protein content and activity. More research is required to determine the cytotoxic activity of soursop seed protein against other cancer cells, as well as its mechanism of action in inducing cancer cell death.

4 Conclusion

Soursop seed protein (*Annona muricata* L.) obtained from Pasar Kleco Surakarta did not have cytotoxic activity against HeLa and Vero cells. Further research is needed to optimize protein isolation techniques so that the active fraction of soursop seed protein has high purity, to evaluate the cytotoxic test method used, and to characterize the active protein of soursop seed.

Acknowledgments. The authors would like to express their gratitude to Universitas Muhammadiyah Surakarta for funding this research through "*Penelitian Unggulan Program Studi* (PUPS)".

Authors' Contributions. All authors contributed to the study's design, experimentation, and statistical analysis. The final manuscript was read and approved by all authors.

References

1. R.M. Zafrial, R. Amalia, Artikel tinjauan : anti kanker dari tanaman herbal, in Farmaka, vol 16, 2018, pp. 15–23. Available from: http://jurnal.unpad.ac.id/farmaka/article/viewFile/17332/pdf
2. F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global Cancer Statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, in CA Cancer Journal for Clinicians, vol 68, 2018, pp 394–424
3. Kementrian Kesehatan RI, Penyakit Kanker di Indonesia Berada Pada Urutan 8 di Asia Tenggara dan Urutan 23 di Asia – P2P Kemenkes RI, 2019, (cited 2021 Dec 22). Available from: http://p2p.kemkes.go.id/penyakit-kanker-di-indonesia-berada-pada-urutan-8-di-asia-tenggara-dan-urutan-23-di-asia/
4. S. Khairani, S.A. Keban, M. Afrianty, Evaluation of drug side effects chemotherapy on Quality of Life (QOL) breast cancer patients at hospital X in Jakarta, in Jurnal Ilmu Kefarmasian Indonesia, vol 17, 2019, pp. 9–13.
5. A. Muheem, F. Shakeel, M.A. Jahangir, M. Anwar, N. Mallick, G.K. Jain, et al. A review on the strategies for oral delivery of proteins and peptides and their clinical perspectives. in Saudi Pharmaceutical Journal, vol 24, 2016, pp. 413–28. DOI: http://dx.doi.org/10.1016/j.jsps.2014.06.004
6. I. Kimiz-Gebologlu, S. Gulce-Iz, C. Biray-Avci, Monoclonal antibodies in cancer immunotherapy. in Molecular Biology Reports, vol 45, 2018, pp. 2935–40. DOI: http://dx.doi.org/10.1007/s11033-018-4427-x

7. M. Zeng, M. Zheng, D. Lu, J. Wang, W. Jiang, O. Sha, Anti-tumor activities and apoptotic mechanism of ribosome-inactivating proteins, in Chinese Journal of Cancer, vol 34, 2015, pp. 1–10.
8. J. Ling, W.Y. Liu, Wang TP, Cleavage of supercoiled double-stranded DNA by several ribosome-inactivating proteins in vitro, in FEBS Letters, vol 345, 1994, pp. 143–146.
9. F. Stirpe, M.G. Battelli, Ribosome-inactivating proteins: progress and problems. in Cellular and Molecular Life Science, vol 63, 2006, pp. 1850–1866.
10. Sismindari, A. Hussana, S. Mubarika. In vitro cleavage of supercoiled double stranded DNA by crude extract of *Annona squamosa* L, in Majalah Farmasi Indonesia, vol 9, 1998, pp. 146–152.
11. S.R. Dhanraj, J.J. Vennila, M. Dhanraj, Pharmacological investigation of ribosome-inactivating protein (RIP) – like protein extracted from *Annona squamosa* L. seeds, in Journal of King Saud University - Science, vol 32, 2020, pp. 2982–2988. DOI: https://doi.org/10.1016/j.jksus.2020.08.002
12. P. Indrayudha, A.R.T. Wijaya, S. Iravati, Uji aktivitas ekstrak daun dewandaru dan daun keladi tikus terhadap pemotongan DNA superkoil untai ganda, in Journal Farmasi Indonesia, vol 3, 2006, pp. 63–70.
13. N.P. Ariantari, Z. Ikawati, Sudjadi, Sismindari, Efek sitotoksik protein dari daun *Mirabilis jalapa* L. hasil pemurnian dengan kolom CM Sepharose CL-6B terhadap kultur sel HeLa, in Farmasains Jurnal Farmasi dan Ilmu Kesehatan, vol 1, 2010.
14. CCRC, Prosedur Tetap Uji Sitotoksik Metode MTT, (cited 2021 Dec 29), Available from: https://ccrc.farmasi.ugm.ac.id/?page_id=240
15. S.C. Tan, B.C. Yiap, DNA, RNA, and protein extraction: the past and the present, in Journal of Biomedicine and Biotechnology, 2009, pp. 1–10.
16. K.C. Duong-Ly, S.B. Gabelli, Salting out of proteins using ammonium sulfate precipitation, in Methods in Enzymology, 2014, pp. 85–94.
17. O. Coskun. Separation techniques: chromatography, in Northern Clinic of Istanbul vol 3, 2016, pp. 156–60.
18. P.M. Cummins, K.D. Rochfort, B.F. O'Connor, Ion-exchange chromatography: basic principles and application philip. in Protein Chromatography, vol 1485, 2017, pp. 209–223.
19. O.B. Acikara, Ion-exchange chromatography and its applications, In: D.F. Martin, B.B. Martin, editors, Column Chromatography, Rijeka: Intech Open, 2013, pp. 31–57.
20. H. Lodish, A. Berk, S.L. Zipursky et al. Molecular Cell Biology 5th Ed, 5th ed. W.H. Freeman, New York: W H Freeman & Co, 2003, Available from: https://www.ncbi.nlm.nih.gov/books/NBK21475/%5Cnhttp:/books.google.com/books/?hl=en&lr=&id=K3JbjG1JiUMC&pgis=1
21. Ismaun, Muzuni, N. Hikmah, Molecular detection of *Escherichia coli* bacteria as a cause of diarrhic disease using techniques of PCR, in Bioma, vol 6, 2021, pp. 1–9.
22. G. Lucena-Aguilar, A.M. Sánchez-López, C. Barberán-Aceituno, J.A. Carrillo-Ávila, J.A. López-Guerrero, R. Aguilar-Quesada, DNA source selection for downstream applications based on DNA quality indicators analysis, in Biopreservation and Biobanking, vol 14, 2016, pp. 264–270.
23. E. Layne, Spectrophotometric and turbidimetric methods for measuring proteins, in Methods in Enzymology, vol 3, 1957, pp. 447–454.
24. A.B. Mahardika, S. Wahyuono, M.S.H. Wahyuningsih, Sitotoksisitas senyawa hasil isolasi daun *Tithonia Diversifolia* (Hemsley) A. Gray) terhadap sel T47D, MCF7 dan EVSA-T, in Journal Majalah Farmaseutik, vol 12, 2016, pp. 401–410.
25. L. Arifianti, Sukardiman, H. Studiawan, Rakhmawati, L. Megawati, Uji aktivitas ekstrak biji sirsak (*Annona muricata* L.) terhadap sel kanker mamalia secara in vitro, in Jurnal Farmasi dan Ilmu Kefarmasian Indonesia, vol 1, 2014, pp. 63–66.

26. Haryoto, Muhtadi, P. Indrayudha, T. Azizah, A. Suhendi, Aktivitas sitotoksik ekstrak etanol tumbuhan sala (*Cynometra ramiflora* Linn) terhadap sel HeLa, T47D dan WiDR, in Jurnal Penelitian Saintek, vol 18, 2013, pp. 21–28.
27. N.H. Nguyen, Q.T.H. Ta, Q.T. Pham, T.N.H. Luong, et al, Anticancer activity of novel plant extracts and compounds from *Adenosma bracteosum* (Bonati) in human lung and liver cancer cells. in Molecules, vol 25, 2020, pp. 1–16.
28. K. Johung, E.C. Goodwin, D. DiMaio, Human Papillomavirus E7 repression in cervical carcinoma cells initiates a transcriptional cascade driven by the retinoblastoma family resulting in senescence, in Journal of Virology, vol 81, 2007, pp. 2102–2116.
29. K. Shen, C. Shen, L. Chen, X. Chen, Y. Chen, Morphological alterations of vero cell exposed to coplanar PCB 126 and noncoplanar PCB 153, in Environmental Toxicology, vol 27, 2012, pp. 26–31.
30. J.T. DiPiro, G.C. Yee, L.M. Posey, S.T. Haines, T.D. Nolin, V. Ellingrod, Pharmacotherapy: A Pathophysiologic Approach Eleventh Edition. 8th ed. Vol. 23, Dicp. New York: Mc Graw Hill; 2020, p. 95.
31. N.M. Putram, I. Setyaningsih, K. Tarman, Anticancer activity from active fraction of sea cucumber, in Jurnal Pengolahan Hasil Perikanan Indonesia, vol 20, 2017, p. 53.