European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 02, 2020

Cytotoxic Activities and Apoptosis Inducing Mechanism of Kiseueur (Antidesma tetrandrum Blume) Leaves and Bark in Breast Cancer Cell Lines (MCF-7)

Siva Hamdani[1*] ; Chaidir[2] ; Anas Subarnas[3] ; Doni Anshar Nuari[1]
[1]Faculty of Mathematics and Natural Science, Universitas Garut
[2]Faculty of Pharrmacy, Universitas Pancasila
[3]Faculty of Pharmacy, Universitas Padjadjaran

*siva@uniga.ac.id

Abstract. Kiseueur (*Antidesma tetrandrum* blume) is an Indonesian native plant that is potential as an anticancer drug. This study aimed to determine the cytotoxic activities and apoptosis inducing mechanism of the extract and fraction of Kiseueur leaves and bark in breast cancer cell lines (MCF-7). The cytotoxic activity was carried out using the MTS assay. The expression of caspase 3 and caspase 9 pro-apoptotic proteins was observed by Western blot analysis. The results showed that the extract and fraction of Kiseueur leaves and bark had varied cytotoxic activities in breast cancer cell lines (MCF-7). Kiseueur bark extract had the highest cytotoxic activity against MCF-7 cells with IC_{50} 81.65 µg / mL. Kiseueur bark extract induced apoptotic mechanism through modulation of caspase 9 and caspase 3 protein expression.
Keywords: **Cytotoxic activity, Apoptosis, MCF-7, *Antidesma tetrandrum***

1. Introduction

Cancer is a group of diseases that involve abnormal cell growth. In all types of cancer, cells continue to divide excessively and have the potential to invade other tissues (1). Cancer causes morbidity and mortality worldwide, wherein every one out of six deaths is caused by cancer (2). Data from the Globocan-IARC (International Agency for Research on Cancer) reported that in 2012, the estimated occurrence of breast cancer cases in the world reached around 1.6 million with 14.7% mortality rate (2). According to the 2013 Riskesdas, in Indonesia, breast cancer is a type of cancer with the second highest incidence with an estimated 61,682 cases. Generally, anticancer chemotherapy drugs targets are to influence signal transduction, signals that regulate cell cycles, growth factors and receptors, DNA repair, and apoptosis (3). Apoptosis is a programmed cell death and involves a series of processes mediated by protease groups, known as cysteinyl aspartic acid-protease (caspase) (4). Research in the process of apoptosis in cancer cells has become a therapeutic approach to various types of cancer. The research is intended to activate the mechanism of induction of apoptosis in cancer cells (3).

Chemotherapy drugs are highly effective against a wide range of cancers, but they have such limitations as a side effect, expensive, very complex, toxic and in some case, there is resistance to chemotherapy drugs. Searching for new anticancer from natural compounds has become promising (5). Antidesma is a genus of tropical plants that belong to the Phyllanthaceae family. Antidesma sp has been well-known in Thailand, India, and Indonesia as plants that have health benefits (6). Research on several plants included in the Antidesma genus showed biological effects, for example, the antibacterial activity shown by *Antidesma madagascariensis* (7), high cytotoxic activity against lung cancer cell line (A549 and COR L23) (6) by *Antidesma thwaitessianum* and *Antidesma acidum*. Other cytotoxic effects are shown by *Antidesma bunius* (L) spreng against Hela cells(8) and *Antidesma pentandrum* against breast cancer cells (MCF-7) and central nerve cancer cells (SF-268) (9).

Antidesma tetrandrum, known as the Kiseueur in West Java or Pelangas in Riau, is one of the species found in the Gunung Salak region (10), Gunung Gede Pangrango Park(11), Cisujen Sukabumi

forest area, West Java (12) and Bukit National Park Tigapuluh Riau. Based on the anti-cancer prescreening test of the *Antidesma tetrandrum* bark with the Brine Shrimp Lethality Test (BSLT) method, the results showed that the species was believed to be cytotoxic with LC_{50} of 19.7 µg/ml (10). Research on the biological activity of Antidesma tetrandrum, especially anticancer, is relatively unknown. Using chemotaxonomy and the results of anticancer screening tests are possible that Kiseueur (*Antidesma tetrandrum*) plant contains cytotoxic compounds and has the potential to be developed as anticancer drugs. Therefore it is essential to evaluate the anticancer activity and its mechanism from the *Antidesma tentrandrum*.

2. Methods

This study aimed to determine the cytotoxic activity and apoptosis inducing mechanism of the extract and fraction of Kiseueur leaves and bark in breast cancer cell lines (MCF-7). The sample was the ethanol extracts and fractions of the Kiseueur leaves and bark obtained from the Center for Plant Conservation Botanical Gardens-LIPI, Bogor. Cytotoxic test was performed using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxphenyl)-2-(4-sul-fophenyl)-2H-tetrazolium assay. The selected (most toxic) extract or fraction will be the sample for inducing apoptosis mechanism that was observed by the Western Blot method. Apoptosis‑associated proteins were analyzed by immunoblot analysis using caspase 3, and caspase 9 whereas GAPDH served as the loading control.

The cytotoxic test was carried out at the Laboratory of Cell and Molecular Biology, Faculty of Pharmacy, University of Padjadjaran. The observation of protein expression was done in the Central Laboratory of the University of Padjadjaran.

3. Result and Discussion

3.1 Result

3.1.1 Phytochemical screening with specific reagents. Phytochemical screening was carried out to determine the secondary metabolite groups contained in the plant. Phytochemical screening results with specific reagents are presented in the following table.

Table 1. *A. tetrandrum* phytochemical screening results

Chemical Content	Leaf Extract	Bark Extract
Alkaloids	-	+
Flavonoids	-	+
Saponin	-	+
Polyphenols	+	+
Tanin	+	+
Quinon	+	+
Steroids / Triterpenoids	+	+

Notes: (+) = detected (-) = undetected

There are chemical compound differences between *A. tetrandrum* leaf and bark extract. Alkaloids, flavonoids and saponin were not detected in the leaf extract.

3.1.2 Thin Layer Chromatography. Thin Layer Chromatography (TLC) pattern monitoring aims to monitor the compounds by polarity-based separation. The results of TLC monitoring are shown in the following figure:

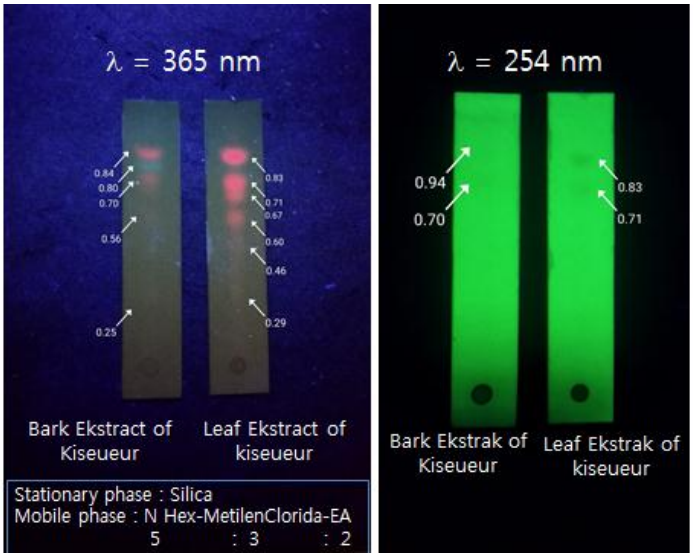

Figure 1. TLC pattern of Kiseueur leaf and bark

TLC was carried out on the ethanol extract of the Kiseueur (*Antidesma tetrandrum*) leaves and bark with the stationary phase: silica gel and the mobile phase using n-hexan: methylene chloride: ethyl acetate (5: 3: 2), observed under UV light of λ 254 nm and λ 365 nm.

3.1.3 High Performance Liquid Chromatography

This study performed HPLC with a Diode Array Detector (DAD). The Analysis of the extracts with HPLC showed that *A. tetrandrum* leaf extract had 56 compounds, and stem bark extract had 67 compounds .This result was in accordance with phytochemical screening wherein the compound in the bark were higher than that of leaf extracts. The UV spectrum exhibited peak with the highest signal intensity of leaf and bark extract are not-too-different- in retention time; 18.83 minutes on leaf extract and 19.33 minutes on bark extract. The two peaks were believed as the markers for *A. tetrandrum.*

3.1.4 Cytotoxicity in breast cancer cells (MCF-7)

Cytotoxicity testing was carried out on the extract and fraction of Kiseueur leaves and bark against MCF-7 breast cancer cells using the MTS Assay method. The IC_{50} value of the extract and fraction of *A.tetrandrum* leaves and bark on MCF-7 are displayed as follows:

Table 2. IC_{50} value of extract and fraction of kiseueur leaf on MCF-7

	Sample	IC_{50} (MCF-7) µg/mL
Leaves	Leaf extract	179,3
	Leaf n-hexane fraction	262,5
	Leaf ethyl acetate fraction	137,5
	Leaf water fraction	206,5
Bark	Stem bark extract	81,6
	Bark n-hexane fraction	112,8
	Bark ethyl acetate fraction	142,8
	Bark water fraction	161,5

Table 2 showed that the most active extract was taken from Kiseueur bark extract (81.65 µg / mL) so that bark extract was used as the selected sample and then tested for the molecular level apoptosis mechanism by observing caspase 3 and caspase 9 protein expression using the western blot method.

3.1.5 Proapoptosis activity. The Western Blot analysis on MCF-7 breast cancer cells that have been treated with *A. tetrandrum* bark extract showed the presence of pro-apoptotic specific protein

expression for caspase initiator, procaspase 9 active fragment at 47-51 kDa and cleaved caspase 9 at 35-40 kDa.(Fig.2)

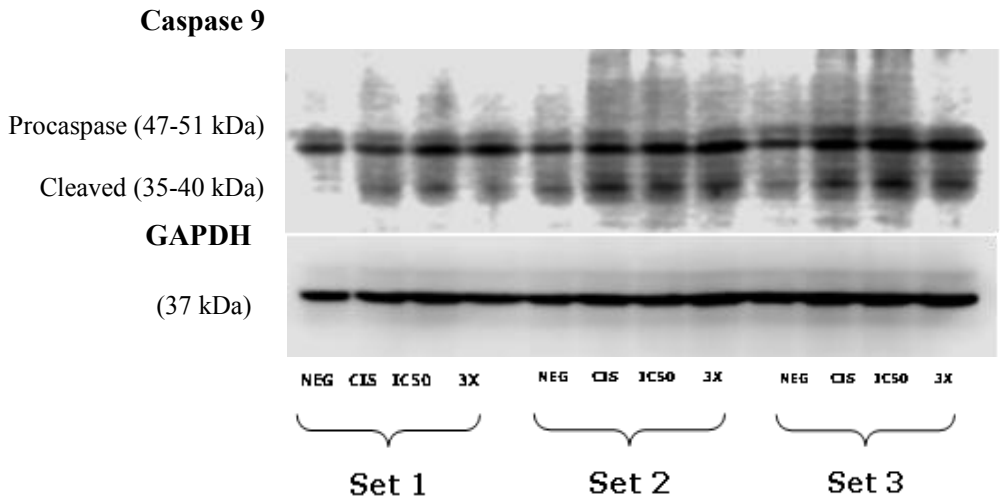

Figure 2. Western blot analysis the protein expression of caspase 9 and GAPDH. Cells treated with Kiseueur bark extract with IC_{50} doses, 3x IC_{50} doses and ciplastin

Cleaved caspase 9 has been formed at both the IC_{50} and 3x IC_{50} doses with a thickness similar to cisplastin. It provides evidence that the bark extract can induce apoptosis through activation of procaspase 9 and then caspase 9 as an initiator caspase (13) in MCF-7 breast cancer cells.

Western blot analysis also showed pro-apoptotic specific protein expression for the caspase executor. Active cleaved caspase 3 fragment observed at 17-19kDa (Fig 3).

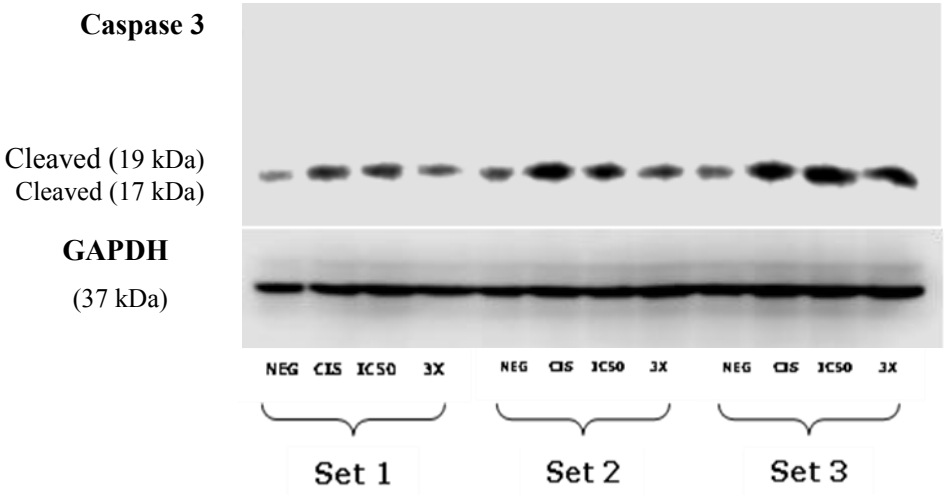

Figure 3. Western blot analysis the protein expression of caspase 3 and GAPDH. Cells treated with Kiseueur bark extract with IC_{50} doses, 3x IC_{50} doses and ciplastin.

It is seen that there are cleaved caspase 3 expressions on IC_{50} and 3 x IC_{50}. It proves that in MCF-7 treated with *A. tetrandrum* bark extract can induce apoptosis through activation of caspase 3 as the executor caspase (13).

3.2. Discussion

Kiseueur (*Antidesma Tetrandrum*) is thought to be cytotoxic and has the potential to be developed into an anticancer drug. Thus far, the information about the phytochemical content of *A. tetrandrum* species less than the other species on the same genus. The results of phytochemical screening obtained different results from that of previous studies. *A. tetrandrum* leaves are known contain alkaloid cyclopeptide (14). Phytochemical screening with specific reagents is rough and non-specific so that leaf extract need to be further studied with other methods. *A. tetrandrum* bark, the

phytochemical screening results are in line with some previous studies where the Kiseueur (*A. tetrandrum*) bark extract was detected to contain chemical compounds from alkaloid, flavonoid, and tannin groups with strong intensity and contained quinones, triterpenoids, and saponins (10).

The chromatogram profile with HPLC showed that the compound components in the *A. tetrandrum* bark are higher than that of the leaves. The UV spectrum exhibited peak with the highest signal intensity of leaf and bark extract are not-too-different- in retention time that the peaks were believed as the markers for *A. tetrandrum*.

Based on IC_{50} values, the cytotoxicity level of extracts can be divided into strong (<100 µg / mL), moderate (101-200 µg / mL), and low (> 200 µg / mL) (15). The cytotoxic test results from extracts and fractions of both leaves and bark were varied, with the bark extract having the smallest IC_{50} level (IC_{50} = 81.6 µg / mL) classified as strong cytotoxicity.

Apoptosis is a mechanism of programmed cell death (1)(16). The apoptotic pathway involves a series of positive and negative regulators mediated by a group of proteases; Cysteinyl Aspartic Acid-Protease (caspase) (4). There are commonly known extrinsic and intrinsic pathways at the stage of apoptosis (17). Both intrinsic and extrinsic pathways are interrelated, and meet at one and the same terminal point called the execution stage (13). Apoptosis that occurs via the intrinsic pathway is stimulated by changes in the mitochondrial membrane with the effect of opening the Mitochondrial Permeability Transition (MPT) gap, loss of mitochondrial transmembrane potential and the release of two main groups of pro-apoptotic proteins from the inter-membrane space into the cytosol, including cytochrome c, Smac / DIABLO, and serine HtrA2 / Omi protease. Cytochrome c binds and activates Apaf-1 and procaspase 9 and forms apoptosome. Furthermore, caspase-9 will activate downstream procstreamase-3. As the executor of proteins, active caspase-3 will break down various types of substrate, including enzymes DNA repair, cellular, and nucleus structural proteins, including the core mitotic apparatus, the lamina nucleus, and other cellular constituents(13)(16). The discovery of procaspase 9 and caspase 9 has been suspected that the trigger for apoptosis by Kiseueur bark extract on MCF-7 breast cancer cells is through the intrinsic pathway.

4. Conclusions

The extract and fraction of Kiseueur (*Antidesma tetrandrum* blume) leaves and bark have varied cytotoxic activity against MCF-7 breast cancer cells with the most cytotoxic level is bark extract (IC_{50} 81.65µg /mL). Kiseueur bark extract inhibited the proliferation of MCF-7 cells through the apoptosis pathway marked by protein expression modulation of caspase 9 and caspase 3.

Acknowledgements

I would like to express my gratitude to Dr. rer.nat Chaidir, Apt. and Professor Anas Subarnas, my research supervisor, for their constructive suggestion and patient guidance. I would also like to extend my thanks to the technicians of the laboratory Universitas Padjajaran who helped me handling the instruments.

References

1. Campbell, N.A., J.B. Reece & LGM. Biologi. 8th ed. Manulu W, editor. Jakarta: Erlangga; 2010.
2. WHO. Cancer fact sheet. WHO media center - Cancer fact sheet. 2017. p. 1.
3. Hassan M, Watari H, Abualmaaty A, Ohba Y, Sakuragi N. Apoptosis and Molecular Targeting Therapy in Cancer. 2014;2014.
4. Ruddon WR. Cancer Biology. 4th ed. NewYork: Oxford University Press; 2007. 143-157 p.
5. Iqbal J, Abbasi BA, Mahmood T, Kanwal S, Ali B, Khalil AT. Plant-derived anticancer agents: A green anticancer approach. Asian Pac J Trop Biomed. 2017;1–23.
6. Tuy-on T. Biological activities of the ethanolic extract from Antidesma twaitessianum and Antidesma acidum rettz for cancer tratment. Faculty of Medichine Thammasat University; 2011.
7. Narod FB, Gurib-Fakim A, Subratty AH. Biological investigations into Antidesma madagascariense Lam. (Euphorbiaceae), Faujasiopsis flexuosa (Lam.) C. Jeffrey (Asteraceae),

Toddalia asiatica (L.) Lam. and Vepris lanceolata (Lam.) G. Don (Rutaceae). J Cell Mol Biol. 2004;3:15–21.
8. Puspitasari E, Umayah Ulfa E. Uji Sitotoksisitas Ekstrak Metanol Buah Buni (Antidesma bunius (L) Spreng) terhadap Sel Hela Cytotoxicity Effect of Methanolic Extract of Buni's Fruits (Antidesma bunius (L) Spreng) against Hela Cells. 2009;10(2):181–5.
9. Yun-Cang Chen, Ming-Jen Cheng S-JL et al. Coumarinolignans from the Root of Formosan Antidesma pentandrum var. Helv Chim Acta. 2004;87(11):2805–2801.
10. Sari RK, Syafii W, Azizah N, Fadli M. Potensi Ekstrak Kulit Kayu dari Hutan Gunung Salak sebagai Agen Antidiabetes dan Antikanker (Potential Antidiabetic and Anticancer Agents from the Inner bark Extractives of Mount Salak Forest Woods). 2014;12(2).
11. Wihermanto W. Dispersion pattern interspecific association and population status of threatened plants on submontane and montane zones of Mount Gede-Pangrango National Park. Biodiversitas, J Biol Divers. 2004;5(1):17–22.
12. Susilo A. Keragaman tumbuhan dan potensi pemanfaatannya di kawasan hutan alam sekunder RPH Cisujen KPH Sukabumi, Jawa Barat. In: Seminar Nasional Masyarakat Biodiversivitas Indonesia. 2016. p. 256–62.
13. Rastogi RP, Richa, Sinha RP. Apoptosis: Molecular mechanisms and pathogenicity. EXCLI J. 2009;8:155–81.
14. Buske A, Hoffmann P. Chemotaxonomy of the tribe Antidesmeae (Euphorbiaceae): antidesmone and related compounds. 2002;60:489–96.
15. Lesmana R, Goenawan H. Fisiologi Molekuler seri prosedur dan protokol laboratorium : Western Blot. Lesmana R, editor. Bandung: Universitas Padjadjaran; 2017.
16. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol. 2007;35(4):495–516.
17. Ghavami S, Shojaei S, Yeganeh B, Ande SR, Jangamreddy JR, Mehrpour M, et al. Autophagy and apoptosis dysfunction in neurodegenerative disorders. Prog Neurobiol. 2014;112:24–49.