Open Veterinary Journal, (2024), Vol. 14(10): 2628-2633
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)

Research Article
DOI: *10.5455/OVJ.2024.v14.i10.12*

Submitted: 07/07/2024 Accepted: 13/09/2024 Published: 31/10/2024

Stem bark ethanolic extract of *Pinus merkusii* induces caspase 9-mediated apoptosis in HeLa cells

Agung Budianto Achmad[1]*, Annise Proboningrat[2], Arif Nur Muhammad Ansori[3], Amaq Fadholly[4], Siti Eliana Rochmi[1], Rinza Rahmawati Samsudin[5], Nanik Hidayatik[6], Gracia Angelina Hendarti[7] and Shara Jayanti[8]

[1]*Department of Health, Faculty of Vocational Studies, Universitas Airlangga, Surabaya, Indonesia*
[2]*Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia*
[3]*Postgraduate School, Universitas Airlangga, Surabaya, Indonesia*
[4]*Division of Pharmacology and Toxicology, School of Veterinary and Biomedical Medicine, IPB University, Bogor, Indonesia*
[5]*Department of Chemistry, Faculty of Health Sciences, Muhammadiyah University of Surabaya, Surabaya, Indonesia*
[6]*Division of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia*
[7]*Division of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia*
[8]*Marine and Fisheries Polytechnic, Surabaya, Indonesia*

Abstract

Background: Cervical cancer is a severe concern for women throughout the world. This percentage of cancer incidence causes sufferers to die at a high rate. It is believed that the bark of the *Pinus merkusii* tree contains anti-cancer compounds that inhibit cervical cancer cell growth.

Aim: This present study aims to examine the cytotoxic ability of *P. merkusii* tree bark ethanol extract (PMBE) by inducing apoptosis in HeLa cells.

Methods: We administered the PMBE at concentrations of 50, 100, 200, and 400 µg/ml to HeLa cell cultures. We then conducted the MTT cytotoxicity assay, detected apoptosis via Annexin V binding, and observed caspase 9 expression via immunocytochemistry.

Results: PMBE showed cytotoxic activity on HeLa cells with an IC_{50} of 226.6 µg/ml for 24 hours of treatment. PMBE caused early apoptosis in up to 81.31% of HeLa cells, as well as increased caspase-9 expression.

Conclusion: Based on this study, PMBE is predicted to have dose-dependent antiproliferative or cytotoxicity effects on the HeLa cell line through the intrinsic pathway apoptosis mechanism.

Keywords: Anticancer, Cervical cancer, Apoptosis, Pinaceae.

Introduction

Cervical cancer ranks fourth worldwide in terms of cancer-related mortality among women; over 85% of cases and deaths from the disease occur in developing countries (Proboningrat *et al*., 2019). Cervical cancer develops as a result of aberrant cell proliferation in the cervix (the lowest part of the uterus that terminates in the vagina) (Sharma *et al*., 2017). The cancer treatment process still uses many surgical procedures, which still have the potential to leave behind cancer cells that can grow again and metastasize to other body organs (Wijaya and Muchtaridi, 2017). Treatment with conventional chemotherapy methods often has negative impacts on the patient's health due to systemic toxicity and drug resistance (Proboningrat *et al*., 2021b). Based on this problem, looking for anti cancer agents based on natural ingredients with low side effects and a better ability to kill cells is necessary. There are many side effects in cancer therapy with conventional models of treatment; it is essential to explore new candidates that are effective with lower side effects against cervical cancer.

Several compounds in plants have anti-cancer properties (Zarrinnahad *et al*., 2018), one of which is *Pinus merkusii* (Proboningrat *et al*., 2021a). *Pinus merkusii* is a member of the Pinaceae family, which is indigenous to Southeast Asia and extensively dispersed throughout Indonesia, Philippines, Laos, Vietnam, Cambodia, Thailand, and Burma (Proboningrat *et al*., 2021a). Pine trees are utilized as a folk cure in Asia

***Corresponding Author:** Agung Budianto Achmad. Department of Health, Faculty of Vocational Studies, Universitas Airlangga, Surabaya, Indonesia. Email: *ab.achmad@vokasi.unair.ac.id*

for gastrointestinal issues, skin and topical conditions, inflammation, ulcers, itching, and snakebites (Sajid *et al*., 2018). The phytochemicals found in the pine plants, including polyphenols, flavonoids, alkaloids, triterpenes, triterpenoids, sterols, glycosides, lignans, and saponins, have been demonstrated to be responsible for the medicinal properties of the plant (Sudjarwo *et al*., 2018).

However, its complete anticancer potential against HeLa cells is still unknown. Previous research (Proboningrat *et al*., 2021a) used the WIDR cell line showed that *P. merkusii* crude extract was potently to killing cancer cells. Apoptosis is one of how cancer cells die through programmed cell death. It works by a cascade series involving increased activity of pro-apoptotic proteins (Bax) (Li *et al*., 2011), decreased expression of the anti-apoptotic protein (Bcl-2), and activation of Caspase 9 and 3 (Chu *et al*., 2012; Liu *et al*., 2015). Thus, in the current study, we assessed the cytotoxicity of the stem bark extract of *P. merkusii* (PMBE) against cervix cancer, HeLa cell line, and the possible mechanism of action by inducing apoptosis via caspase-9 activation.

Materials and Methods

Reagents

Ethanol 96%, Dulbecco's modified eagle medium (Gibco, USA), 2% penicillin-streptomycin (Gibco, USA), amphotericin B (Sigma-Aldrich, USA), HEPES (Sigma-Aldrich, USA), 10% fetal bovine serum (Rocky Mountain Biologicals, Inc., USA), 1 × phosphate buffer saline (PBS) (Sigma-Aldrich, USA), dimethyl sulfoxide (SigmaAldrich, USA), Trypsin/EDTA solution (Sigma-Aldrich, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA), HCl 0.1 N (Sigma-Aldrich, USA), sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA), Annexin V apoptosis detection kit with PI (BioLegend Inc., UK), anti-caspase-9 antibody (ab52298, Abcam), and Mayer's hematoxylin solution (Sigma-Aldrich, USA).

Plant extract preparation

The stem barks of *P. merkusii* were gathered from Malang Regency, East Java Province, Indonesia. After being cleansed, the dried stem barks were chopped into tiny pieces. After that, they were ground into a powder. 350 g of powdered bark were steeped for 3 days in 1.75 l of 96% ethanol. A rotary evaporator running at 250 rpm and 60°C separated and concentrated the macerate (Proboningrat *et al*., 2021a).

Cell culture

The Department of Parasitology of Universitas Gadjah Mada's School of Medicine contributed to the HeLa cell lines. The cells were cultured at 37°C with 5% CO2 in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, HEPES, 0.5% amphotericin B, and 2% penicillin-streptomycin.

MTT cytotoxicity assay

HeLa cells were seeded at a density of 104 cells/ml, and after being incubated overnight, they were exposed to a range of concentrations of PMBE starting with the highest concentration of 400 µg/ml (four folded dilution) for 24 hours. After incubation, 100 µl of MTT solution was added and left in each well for 4 hours. The absorbance was measured at a wavelength of 595 nm using a Benchmark Microplate Reader (Bio-Rad, USA) following the addition of 100 µl of SDS 10%. Graphs were plotted to find the percentage of live cells in a particular value.

Apoptosis detection by annexin V binding

Detection of the cells' apoptosis was performed using the FITC Annexin V apoptosis detection kit with PI. HeLa cells were plated in 6-well plates at a density of 106 cells/well. After being incubated overnight (37°C, 5% CO2), they were treated with IC_{50}, $2IC_{50}$, and $4IC_{50}$ of PMBE for 24 hours. Following harvesting, the cells were rinsed with PBS, re-suspended in Annexin V binding buffer, and stained for 15 minutes at room temperature in the dark with 5 µl of Annexin V and 10 µl of PI. The distribution of cell populations in various quadrants was identified using the BD FACSCalibur flow cytometer (BD Biosciences, USA).

Caspase-9 detection by immunocytochemistry

HeLa cells (2 × 104 cells/well) were plated and incubated overnight on coverslips within 24-well plates at 37°C and 5% CO2 prior to being treated with IC_{50}, $2IC_{50}$, and $4IC_{50}$ of PMBE for 24 hours. After 10 minutes of fixation using absolute methanol, the cells were washed with PBS and treated with a 1% hydrogen peroxide solution for 10 minutes. They were then blocked with a background sniper and incubated with a 1:50 anti-caspase-9 antibody in a dark room for 1 hour. After washing, the Star Trek Universal HRP Detection System was added, and Mayer's hematoxylin was used for counterstaining. The cells were dipped in ethanol, dried, and mounted. The stained cells were observed using a Nikon Eclipse Ci microscope (Nikon Corp., Japan) and evaluated semi-quantitatively by the H-score method (Mazières *et al*., 2013).

Statistical analysis

Results were analyzed using GraphPad Prism by one-way analysis of variance and Kruskal-Wallis, and differences were deemed statistically significant at the level of p-values ≤ 0.05.

Ethical approval

Not needed for this study.

Results

Cytotoxicity effects of PMBE on HeLa cells

We examined the ethanolic bark extract of PMBE to determine its capacity to inhibit the cervical cancer HeLa cell's growth or viability to verify the antiproliferative effect of the plant. Initially, an MTT assay was conducted using a range of dosages applied to the cells, starting from 400 µg/ml to 200, 100, 50, and

25 µg/ml. As shown in Figure 1, PMBE demonstrated a dose-dependent ability to inhibit cell growth, with an IC_{50} of 226.6 µg/ml after 24 hours.

Apoptosis induction of HeLa cells by PMBE

To determine whether PMBE is involved in programmed cell death, we assessed the proportion of apoptotic cells in treated HeLa cells. The proportion of early (Annexin V+/PI) and late (Annexin V+/PI+) apoptotic cells shown in Figure 2 indicates the apoptotic cells. The HeLa cells treated with IC_{50} PMBE exhibited the highest proportion of early apoptosis of any group, reaching up to 81.31%. Meanwhile, there was a dose-dependent rise in the percentage of late apoptosis, with the $4IC_{50}$ PMBE group exhibiting the most significant proportion at 3.48%. It was indicated that treating HeLa cells with PMBE for 24 hours incubation tended to cause early apoptosis.

Caspase-9 activation of HeLa cells by PMBE

The dose-course effect of PMBE extract after 24 hours on the initiator caspase-9 activity is depicted in Figure 3. The results indicate that there is an increase in caspase-9 expression in all treated cells compared to untreated cells. Moreover, the results revealed that PMBE induced the potentially highest activity of caspase-9 in HeLa cells exposed to the $2IC_{50}$ concentration. The findings show that PMBE causes HeLa cells to undergo apoptosis by upregulating the expression of caspase-9.

Discussion

In recent decades, scientists have been striving to find and isolate novel anticancer medicines from natural resources. Cytotoxic substances are among the most efficacious anticancer medications (Sarvmeili *et al*., 2016). Some plant active components, such as alkaloids, flavonoids, and phenolic compounds, have been proven to possess cytotoxic activity against cancer cells (Ganadhal *et al*., 2021). Multiple investigations have demonstrated the existence of cytotoxic substances in plants of the Pinaceae family (Shi *et al*., 2016; Yi *et al*., 2016).

Pinus merkusii is a member of the Pinaceae family and is extensively found in Indonesia, particularly in Sumatera and Java Island (Imanuddin *et al*., 2020). Although prior research has conducted cytotoxicity screening of chitosan-based encapsulation *P. merkusii* (Proboningrat *et al*., 2019), there is still a significant lack of scientific data addressing the potential therapeutic effectiveness of the plant's bark's crude extract for cervical cancer and its mechanism of action. According to this study, PMBE extracted from *P. merkusii* decreased the growth of proliferative cells, which was associated with the induction of apoptosis, as indicated by the increase in caspase activity.

Prior studies have already documented the inhibitory activity of various pine plant species on HeLa cell proliferation (Amalinei *et al*., 2014; Li *et al*., 2016; Sarvmeili *et al*., 2016). In the present study, PMBE exhibited a dose-dependent growth inhibitory effect, which triggers cellular processes that suppress proliferation and induce cell death. Our MTT assay confirmed that PMBE inhibited the growth of 50% of HeLa cells at a concentration of 226.6 µg/ml, which was slightly lower than what was reported before (235.60 µg/ml, WiDR cells) (Proboningrat *et al*., 2021a). In addition, it has been documented that bark extracts derived from different species of pine trees are rich in proanthocyanidins, which possess promising

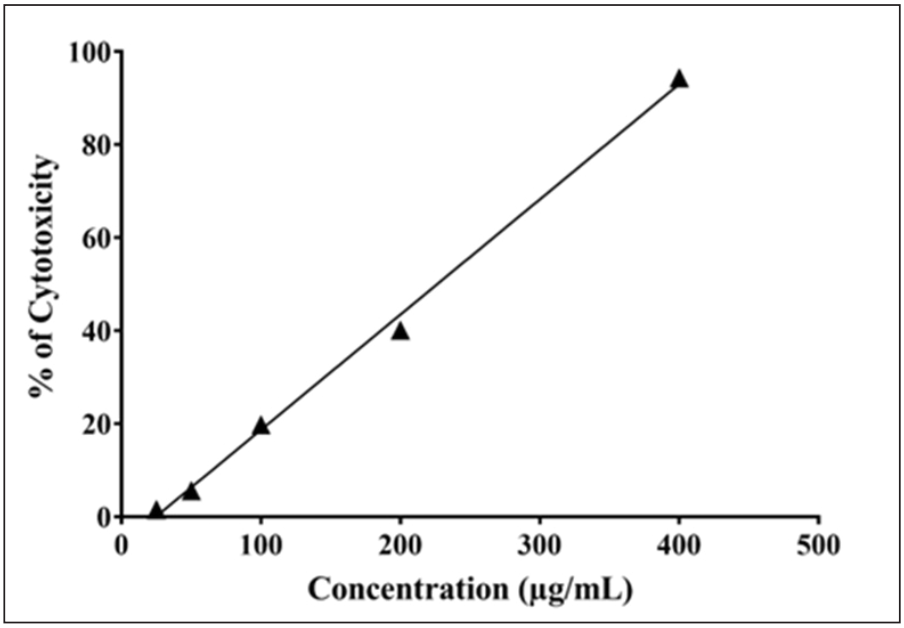

Fig. 1. Cytotoxicity of *Pinus merkusii* stem bark ethanolic extract on HeLa cells.

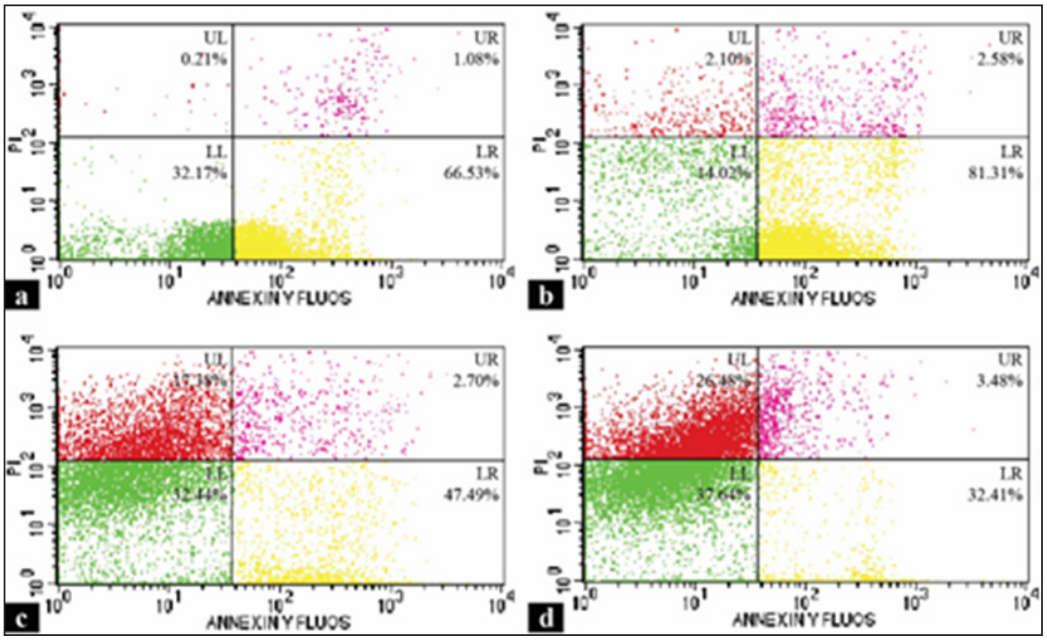

Fig. 2. The flow cytometric histogram shows the activation of apoptosis in HeLa cells after a 24-hour treatment with PMBE. (a) control, (b) IC_{50}, (c) $2IC_{50}$, and (d) $4IC_{50}$. The lower left, lower right, upper right, and upper left, respectively, represent viable, early apoptotic, late apoptotic, and necrotic cells.

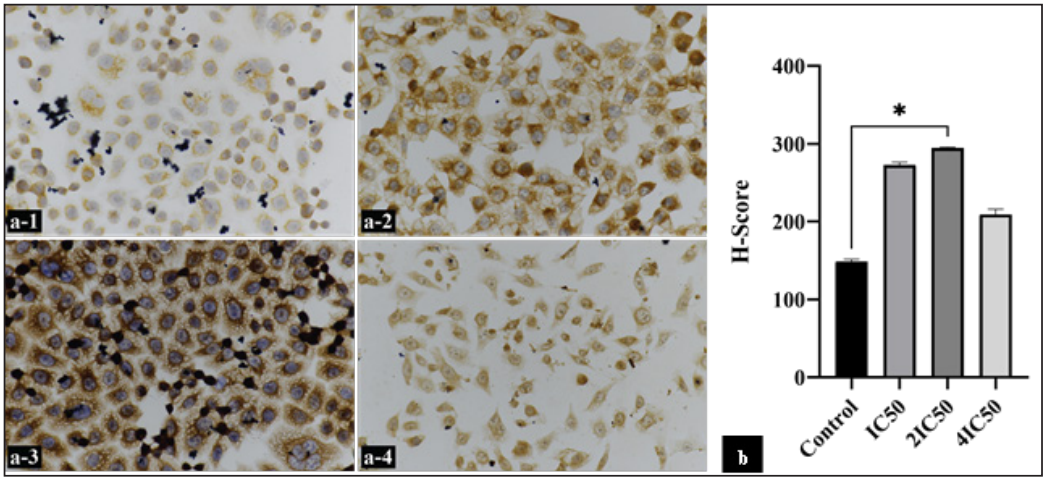

Fig. 3. Caspase-9 expression in (a-1) untreated HeLa cells and HeLa cells treated with (a-2) IC_{50}, (a-3) $2IC_{50}$, and (a-4) $4IC_{50}$ of PMBE for 24 hours (Nikon Eclipse Ci; 400×). (b) The bar displays the mean ± standard deviation of the caspase-9 expression score in treated HeLa cells.

therapeutic properties (Li *et al*., 2015). The potential of proanthocyanidins to inhibit tumor growth has been established on account of their capacity to regulate the activity of multiple targets implicated in carcinogenesis (Rauf *et al*., 2019).

We further investigated the mechanisms by which PMBE inhibits the growth of cervical cancer cells. Apoptosis may be responsible for the inhibitory effect on cancer cell proliferation. Apoptosis plays a significant part in eliminating mutated or rapidly proliferating tumor cells (Millimouno *et al*., 2014). Some studies have revealed that bark extracts from *P. massoniana* and *P. maritima* induce apoptosis in human ovarian cancer A2780 cells and human malignant melanoma A375 cells, respectively (Liu *et al*., 2015; Thaichinda *et al*., 2020). According to flow cytometry results from the current investigation, the proportion of apoptotic cells increased substantially after PMBE treatment. The findings suggest that PMBE triggers apoptosis in cervical cancer cells.

Apoptosis induction is associated with upregulation of pro-apoptotic proteins and downregulation of anti-apoptotic proteins (Singh *et al*., 2015). The Bcl-2 protein is crucial for modulating the mitochondria-mediated caspase activation pathway (Akl *et al*., 2014). When Bcl-2 is down-regulated, it leads to the decline of mitochondrial membrane potential and the release of cytochrome c from mitochondria into the cytosol, which in turn activates caspase-9 (Proboningrat *et al*., 2019). Cleaved caspase-9 additionally stimulates caspase-3, a pivotal enzyme in the intrinsic apoptotic pathway, thereby propelling subsequent apoptotic processes (Moghadamtousi *et al*., 2014). Herein, we observed an increase in caspase-9 expression in HeLa cells treated with PMBE. It could be suggested that PMBE may potentially trigger apoptosis via the mitochondria-associated apoptotic pathway. The proanthocyanidin content commonly present in pine bark extract may be responsible for the PMBE's ability to induce apoptosis in cervical cancer cells. Consistently, Shi *et al*. (2019) documented that proanthocyanidin from grape seeds could trigger apoptosis of human colon cancer SW480 and SW620 cells by facilitating the expression of the pro-apoptotic proteins Bax and Bax, suppressing the expression of the anti-apoptotic protein Bcl-2, and activating caspase-9 and caspase-3.

The results of this study reported that PMBE was predicted to have cytotoxic activities by targeting the mitochondrial-apoptosis pathway in cervical cancer HeLa cell lines. However, further *in vitro* and *in vivo* research is required to improve the findings of this study.

Conclusion

Our study has demonstrated that crude extracts from the bark of PMBE exhibited dose-dependent antiproliferative effects on the HeLa cell line. Furthermore, HeLa cells were induced to undergo apoptosis via a caspase-dependent pathway by the PMBE. Additional research in the field of anti-cancer drug discovery should be conducted, as demonstrated by the current study's findings regarding the potency of PMBE as an anti-cancer agent.

Acknowledgment

The authors thank Gadjah Mada University for providing facilities for this research.

Conflict of interest

All authors declare that there are no conflicts of interest in the present study.

Funding

This work was supported by Airlangga Research Fund, Universitas Airlangga.

Author's contributions

Conceived, designed, and coordinated the study: ABA, AP, and SER. Designed data collections tools, supervised the field sample and data collection, and laboratory work as well as data entry: ANMA, AF, and SJ. Validation, supervision, and formal analysis: RRS and NH. Carried out the statistical analysis and interpretation and participated in the preparation of the manuscript: AP, ABA, and GAH. All authors have read, reviewed, and approved the final manuscript.

Data availability

All data supporting the findings of this study are available within the manuscript and no additional data sources are required.

References

Akl, H., Vervloessem, T., Kiviluoto, S., Bittremieux, M., Parys, J.B., De Smedt, H. and Bultynck, G. 2014. A dual role for the anti-apoptotic Bcl-2 protein in cancer: mitochondria versus endoplasmic reticulum. Biochim. Biophys. Acta. 1843(10), 2240–2252.

Amalinei, R.L.M., Trifan, A., Cioanca, O., Miron, S.D., Mihai, C.T., Rotinberg, P. and Miron, A. 2014. Polyphenol-rich extract from Pinus sylvestris L. Bark-chemical and antitumor studies. Rev. Med. Chir. Soc. Med. Nat. lasi. 118(2), 551–557.

Chu, H.L., Mao, H., Feng, W., Liu, J.W. and Geng, Y. 2012. Effects of sulfated polysaccharide from masson pine (Pinus massoniana) pollen on the proliferation and cell cycle of HepG2 cells. Int. J. Biol. Macromol. 55, 104–108.

Ganadhal, P.C., Nagaraj, K. and Krishna, V. 2021. *In vitro* cytotoxic potential of alkaloid and flavonoid rich fractions of alseodaphne semecarpifolia against MCF-7 cells. Biomed. Pharmacol. J. 14(2), 557–565.

Imanuddin, R., Hidayat, A., Rachmat, H.H., Turjaman, M., Pratiwi, Nurfatriani, F., Indrajaya, Y. and Susilowati, A. 2020. Reforestation and sustainable management of *Pinus merkusii* forest plantation In Indonesia: a review. Forests. 11(12), 1235.

Li, K., Li, Q., Zhang, T., Han, Z., Lian, J., Liu, Z. and Zheng, F. 2011. Procyanidins from *Pinus koraiensis* bark inhibits HeLa cell growth by inducing apoptosis and reducing survivin protein expression. Afr. J. Biotechnol. 10(40), 7766–7771.

Li, Y.Y., Feng, J., Zhang, X.L. and Cui, Y.Y. 2015. Pine bark extracts: nutraceutical, pharmacological, and toxicological evaluation. J. Pharmacol. Exp. Ther. 353(1), 9–16.

Li, Y.Y., Feng, J., Zhang, X.L., Li, M.Q. and Cui, Y.Y. 2016. Effects of Pinus massoniana bark extract on the invasion capability of HeLa cells. J. Funct. Foods. 24, 520–526.

Liu, J., Bai, J., Jiang, G., Li, X., Wang, J., Wu, D., Owusu, L., Zhang, E. and Li, W. 2015. Anti-tumor effect of pinus massoniana bark proanthocyanidins on ovarian cancer through induction of cell apoptosis and inhibition of cell migration. PLoS One. 10(11), e0142157.

Mazières, J., Brugger, W., Cappuzzo, F., Middel, P., Frosch, A., Bara, I., Klingelschmitt, G. and

Klughammer, B. 2013. Evaluation of EGFR protein expression by immunohistochemistry using H-score and the magnification rule: re-analysis of the saturn study. Lung Can. 82(2), 231–237.

Millimouno, F.M., Dong, J., Yang, L., Li, J. and Li, X. 2014. Targeting apoptosis pathways in cancer and perspectives with natural compounds from mother nature. Cancer Prev. Res. 7(11), 1081–1107.

Moghadamtousi, S.Z., Karimian, H., Rouhollahi, E., Paydar, M., Fadeinasab, M. and Kadir, H.A. 2014. Annoa muricata leaves induce G1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-29 colon cancer cells. J. Ethnopharmacol. 156, 277–289.

Proboningrat, A., Ansori, A.N.M., Fadholly, A., Putri, N., Kusala, M.K.J. and Achmad, A.B. 2021a. First report on the cytotoxicity of *Pinus merkusii* bark extract in WiDr, a human colon carcinoma cell line. Res. J. Pharm. Tech. 14(3), 1685–1688

Proboningrat, A., Fadholly, A., Iskandar, R.P.D., Achmad, A.B., Rantam, F.A. and Sudjarwo, S.A. 2019. The potency of chitosan-based *Pinus merkusii* bark extract nanoparticles as anti-cancer on HeLa cell lines. Vet. World 12(10), 1616–1623.

Proboningrat, A., Jayanti, S., Fadholly, A., Ansori, A.N.M., Putri, N., Kusala, M.K.J., Sudjarwo, S.A., Rantam, F.A. and Achmad, A.B. 2021b. The cytotoxicity of ethanolic extract of *Allium cepa* L. on hela cell lines. Res. J. Pharm. Tech. 14(9), 4969–4972.

Rauf, A., Imran, M., Abu-Izneid, T., Ul-Haq, I., Patel, S., Pan, X., Naz, S., Silva, A.S., Saeed, F. and Suleria, H.A.R. 2019. Proanthocyanidins: a comprehensive review. Biomed. Pharmacother. 116, 108999.

Sajid, A., Manzoor, Q., Iqbal, M., Tyagi, A.K., Sarfraz, R.A. and Sajid, A. 2018. Pinus roxburghii essential oil anticancer activity and chemical composition evaluation. EXCLI. 17, 233–245.

Sarvmeili, N., Dehkordi, A.J. and Zolfaghari, B. 2016. Cytotoxic effects of Pinus eldarica essential oil and extracts on HeLa and MCF-7 cell lines. Res. Pharm. Sci. 11(6), 476–483.

Sharma, A., Jyoti, K., Bansal, V., Jain, U.K., Bhushan, B. and Madan, J. 2017. Soluble telmisartan bearing poly (ethylene glycol) conjugated chitosan nanoparticles augmented drug delivery, cytotoxicity, apoptosis and cellular uptake in human cervical cancer cells. Mater. Sci. Eng. C. 72, 69–76

Shi, H., Chengcheng, L.V. and Zhu, Y. 2019. Grape seed proanthocyanidins alters the characteristics of colon cancer cells regarding an Akt signaling. Clin Oncol. 4, 1602.

Shi, X., Liu, D., Zhang, J., Hu, P., Shen, W., Fan, B., Ma, Q. and Wang, X. 2016. Extraction and purification of total flavonoids from pine needles of Cedrus deodara contribute to anti-tumor *in vitro*. BMC Complement. Altern. Med. 16, 1–9.

Singh, L., Pushker, N., Saini, N., Sen, S., Sharma, A., Bakhshi, S., Chawla, B. and Kashyap, S. 2015. Expression of pro-apoptotic bax and anti-apoptotic Bcl-2 proteins in human retinoblastoma. Clin. Experiment. Ophthalmol. 43(3), 259–267.

Sudjarwo, S.A., Wardani, G., Eraiko, K. and Koerniasari, K. 2018. The potency of nanoparticle of *Pinus merkusii* as immunostimulatory on male wistar albino Rat. Int. J. Nutr. Pharmacol. Neurol. Dis. 8(1), 10–15.

Thaichinda, S., Tancharoen, S., Kanekura, T., Higashi, Y., Dararat, P., Kikuchi, K. and Nararatwanchai, T. 2020. Pinus maritima extract induces apoptosis in human malignant melanoma cells via ROS/ Caspase-3 signaling. Nat. Prod. Commun. 15(5), 1–10.

Urbańska, K., Sokołowska, J., Szmidt, M. and Sysa, P. 2014. Review glioblastoma multiforme – an overview. Współczesna Onkol. 18(5), 307–312.

Wijaya, C.A. and Muchtaridi, M. 2017. Pengobatan Kanker melalui Metode Gen Terapi. Farmaka. 15(1), 53–68.

Yi, J., Wang, Z., Bai, H., Li, L., Zhao, H., Cheng, C., Zhang, H. and Li, J. 2016. Polyphenols from pinecones of Pinus koraiensis induce apoptosis in colon cancer cells through the activation of caspase *in vitro*. RSC Adv. 6, 5278–5287.

Zarrinnahad, H., Mahmoodzadeh, A., Hamidi, M.P., Mahdavi, M., Moradi, A., Bagheri, K.P. and Shahbazzadeh, D. 2018. Apoptotic effect of melittin purified from iranian honey bee venom on human cervical cancer HeLa cell line. Int. J. Pept. Res. Ther. 24(4), 563–570.