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Research Article

Cytotoxic Activity of Indonesian *Pogonatum neesii* Dozy from Cibodas Botanical Garden: *In Silico* Molecular Docking and *In Vitro* Evaluation

Rizky Nurdiansyah[1], Agus Budiawan Naro Putra[2], Ainun Nadhifah[3], Erika Chriscensia[4], Ulung Khoe Gondo Kusumo[4], Stephanie Angela Yosiano[5], Anastasia Beatrix Musung[6], Sintikhe A Wenas[6], Steve Makalew[6], Patricia Lovina[4], Intani Quarta Lailaty[7], Fransisca Aurelia Rahmad[8], Fandi Sutanto[4], Pietradewi Hartrianti[4*]

[1]*Department of Bioinformatics, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta 13210, Indonesia.*
[2]*Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research and Innovation Agency (BRIN), Cibinong, 16911, Indonesia.*
[3]*Research Center for Biosystematics and Evolution, National Research and Innovation Agency (BRIN), Cibinong, 16911, Indonesia.*
[4]*Department of Pharmacy, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta 13210, Indonesia.*
[5]*Department of Biotechnology, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta 13210, Indonesia.*
[6]*Department of Biomedicine, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta 13210, Indonesia.*
[7]*Research Center for Plant Conservation, Botanic Gardens and Forestry, National Research and Innovation Agency (BRIN), Bogor 16122, Indonesia*
[8]*Department of Biology, Faculty of Biotechnology, Universitas Surabaya, Surabaya 60293, Indonesia.*

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Abstract

Background: The exploration of bryophytes biodiversity in Indonesia due to its abundance and the bioactivity of its phytochemical content, such as alkaloids and polyphenols, has received increased interest. Despite some species proven to possess pharmacological properties, the antiproliferative study of Indonesian native moss, such as the *Pogonatum* genus, is limited. Hence, this study aims to evaluate the anticancer effects of *Pogonatum neesii* Dozy antiproliferative activity on colon and cervical cancer through *in silico* and *in vitro* methods.

Methods: Molecular docking analysis using Autodock VINA in PyRx softwre was conducted between natural compounds found on *P. neesii* and several target proteins, DNA (cytosine-5)-methyltransferase 1 (DMT-1) (Protein Data Bank (PDB) id: 4WXX) in colon cancer and B-cell lymphoma 2 (Bcl-2) (PDB id: 4LXD) in cervical cancer. Afterwards, total phenolic and alkaloid contents were measured. Subsequently, *P. neesii* was tested on HaCaT (keratinocytes), HEK293 (human embryonic kidney), HT-29 (colorectal cancer models) and HeLa (cervical cancer model) to observe its cytotoxicity.

Results: Out of eight compounds, chlorogenate was found to exert the best binding energy with target proteins, although it had lower binding affinity than the protein's natural ligand. However, the biological, drug-likeness, and toxicity analysis suggested the drug potency of the compound, thus we did the *in vitro* analysis. *P. neesii* showed significant cytotoxic effects on HT-29 and HeLa cells, while it did not exert any cytotoxic effects on HaCaT and HEK-293 cells, at the same concentrations.

Conclusion: *P. neesii* has been shown to have the potential as an anticancer agent through *in silico* and *in vitro* analysis, where the extract showed selective cytotoxicity towards cancer cell lines and cytocompatibility towards normal cell lines. Chlorogenate was pinpointed as the compound with the most activity and interaction with the target proteins in both cancers.

Introduction

Indonesia has abundant natural resources which have proven to provide a lot of benefits to various aspects of life, including health. Approximately 15% of the different plant species of Indonesia are used as traditional medicine.[1] The utilization of natural resources should not be limited only to plants that are commonly cultivated but should also focus on plants that are rarely studied, such as *bryophytes*. Moss plant or *bryophytes* is known to synthesize various phytochemicals to survive in a harsh environment such as high temperature, UV radiation, and various pests and pathogens.[2] They are also able to absorb minute amounts of moisture from mist, dew, and fog that higher plants cannot. Traditionally, bryophytes have been used by tribal people for hair-related diseases and in traditional Chinese medicine to cure fevers, skin diseases, etc.[3] Currently, there

*Corresponding Author: Pietradewi Hartrianti, E-mail: pietradewi.hartrianti@i3l.ac.id

are several studies regarding the use of moss as the source of active compounds, but unfortunately the research on moss plants from Indonesia is still lacking.

Among bryophytes previously studied, the member of the *Pogonatum* genus is one of the most abundant in Cibodas Botanical Garden, Indonesia. It has been previously found that bryophytes possess anticancer activities.[3] Various phenolic and alkaloid compounds have been found in bryophytes[2], which has been strongly linked to anticancer activities *in vitro* and *in vivo*.[4] However, these studies were limited to *in* vitro, without understanding the molecular interaction of the compounds found in the extract. Hence, it is necessary to have a better understanding regarding the *Pogonatum* genus anti-cancer activity. One method that could be used to screen the potential mechanism of the compound in *Pogonatum* genus is through *in silico* molecular docking, which can analyze the binding possibility and affinity between the compound and possible target proteins on the cancer cell.[5] The molecular docking then being followed by absorption, distribution, metabolism, elimination and toxicity (ADME-Tox) as one of the crucial method in drug discovery and provide a quick preliminary screening before the compound is further tested.[6,7]

As the secondary metabolites were hypothesized to be the compounds exerting the anticancer effects, the interactions between these compounds and target proteins found in cancer cells needed to be observed first *in silico*. Therefore in this study, secondary metabolites known to be found in the *Pogonatum* genus were selected from PubChem, including sinapic acid, hydroxycinnamic acid, vanillate, gallate, chlorogenate, ferulate, phloroglucinol and catechol.[8] DNA methyltransferase 1 (DNMT1) was selected as a target protein for cervical cancer, as it has been identified in tumor suppression mechanisms in HeLa cells.[9] On the other hand, B cell lymphoma 2 (Bcl-2) has been shown to play a big role in colorectal cancer apoptosis[10], thus was also chosen as the target protein for colorectal cancer. Furthermore, to confirm their potential as anti-cancer candidate, the total phenolic, and alkaloid contents of *Pogonatum neesii* Dozy, a member of the *Pogonatum* genus, was analyzed and *in vitro* cytotoxicity study was performed against HeLa as a cervical cancer model and HT-29 as colorectal cancer model were performed to confirm their antiproliferative activity. On the other hand, human embryonic kidney cells (HEK293) and human immortalized keratinocytes (HaCaT) cells were used as control cell lines to observe their cytotoxicity against non-cancerous cells.

Methods

In silico analysis

P. neesii natural compounds and target proteins collection

Simplified Molecular Input Line Entry System (SMILES) notation from natural compounds from *P. neesii* was taken from PubChem (https://pubchem.ncbi.nlm.nih.gov/, Accessed 6 July 2022), based on a previous study.[8] A few general phenolics commonly found in *Pogonatum* sp. were 4-hydroxycinnamic acid, vanillate, gallate, chlorogenate, ferulic acid, phloroglucinol, catechol, and sinapic acid.[8] Navitoclax was used as a control towards the target protein for colon cancer and S-adenosyl-L-homocysteine as a control towards the target protein for cervix cancer. The 3D structure from each compound was downloaded in sdf format.

The target protein crystal structure was collected from Protein Data Bank (PDB) web server (https://www.rcsb.org/) (Accessed 6 July 2022). To test the anticancer potential of *P. neesii*, a test on the inhibition capability against commonly found protein in patients suffering from cervix and rectum cancers. Specific protein found in cervix cancer was human DNA (cytosine-5)-methyltransferase 1 (DNMT1) with a PDB code of 4WXX.[11] While the specific protein found in rectum cancer is Bcl 2-navitoclax analog (without thiophenyl) complex with a PDB code of 4LXD.[12]

P. neesii natural compound biological activity analysis

The biological activity analysis of the natural compounds included anticarcinogenic and apoptosis agonist based on probability of activity (Pa value) and probability of inactivity (Pi value) from each compounds using the prediction of activity spectra for substances (PASS) ONLINE web server (http://way2drug.com/passonline/index.php) (Accessed 10 July 2022). This analysis was based on the relation between structure of a compound with the activity that it has.[13]

Pa score is the probability a compound becomes "active", this predicts the probability that the compound that is being studied is categorized in the sub-class of active compound. *Pi* score is the probability to become "inactive", where this predicts the probability that a compound is included in the inactive category.[13]

P. neesii natural compound toxicity analysis

Toxicity analysis of the natural compounds of *P. neesii* was done through web server Protox II (https://tox-new.charite.de/protox_II/) (Accessed 12 July 2022). This was to approximate the dose that caused 50% lethality (LD50) and classification of toxic compounds based on GHS (Globally Harmonized System).[14]

P. neesii natural compound feasibility analysis

Feasibility analysis was performed to calculate the physicochemistry parameter, predict absorption distribution, metabolism and excretion (ADME) parameters, and pharmacokinetics properties, such as drug chemical behaviors that would support drug discovery.[15] SMILE notation from the natural compounds of *P. neesii* which has been obtained from PubChem was analyzed using the Swiss ADME web server (http://www.swissadme.ch/, Accessed 13 July 2022).

Molecular docking and visualization of the P. neesii natural compounds against target proteins

Molecular docking was done by using AUTODOCK VINA inside the PyRx software to discover the binding affinity and strength of binding of the natural compounds found in *P. neesii* with target protein.[16] The docking was performed as specific docking by setting the search grid in the following coordinates: X = 22.4521, Y = 35.9427, Z = 11.8315 for 4LXD and X = -45.5386, Y = 62.8759, Z = 8.6372 for 4WXX, while the rest of the setting was left at default. Binding affinity result and the docking conformation were retrieved for further analysis. Docking conformations were then visualized in 2D binding diagram and 3D conformation visualization to observe the bonding type and quantity between the target protein with the natural compounds. Biovia Discovery Studio Viewer 2021 was used to visualize both.

P. neesii extraction

Fresh *P. neesii* was obtained from Cibodas Botanical Garden, Bogor, Indonesia. Herbarium specimens of *P. neesii* were identified by Dr. Ainun Nadifah were stored in the Cibodas Hortus Tjibodensis (CHTJ), Cibodas Botanical Garden with collection number of NAD 918. The extraction process of the whole plant was carried out in stages using maceration. The powdered plant material (56 g) was extracted three times with 80% ethanol (Merck, USA), 24 h for each extraction cycle. The filtrate was combined and evaporated using a rotary evaporator (R-100 rotavapor, Buchi, Switzerland) and left to dry under fume hood for a few days.

Phytochemical characterization of P. neesii extract

Total phenolic content (TPC)

TFC was conducted using the Folin-Ciocalteu method to measure the total content of phenolic compounds present in the extract based on gallic acid equivalent.[17] As much as 0.08 g of the extract was dissolved in 10 mL methanol and then filtered. The resulting volume was then adjusted to 10 mL with methanol. Standard curve was made using gallic acid in methanol. Five hundred microliters of sample or standard were each mixed with 2.5 mL 7.5% Folin-Ciocalteu reagent and left to react for 8 min. Two milliliters of NaOH 1% were added and the solution was then incubated for 1 h at room temperature prior to absorbance measurement using UV-Vis spectrophotometer (Shimadzu, Japan) at 730 nm. The result was expressed as mg gallic acid equivalent per gram of extract (mg GAE/g extract).

Total alkaloid content (TAC)

TAC was conducted according to previous study with some modifications to measure the total content of phenolic compounds present in the extract based on atropine equivalent.[18] Extract preparation was done by dissolving 50 mg extract in 25 mL methanol. Bromocresol green (BCG) (Merck, USA) solution was prepared by heating 34.9 mg BCG with 1.5 mL 2N NaOH (Merck, USA) and 2.5 mL distilled water until completely dissolved. Then, the solution was diluted to 500 mL with distilled water. Five hundred microliters of each dissolved plant extract and standard solution was mixed with 500 µL 2N HCl respectively. Two and a half mL BCG was added, along with 2.5 mL 2 M phosphate buffer (Merck, USA) pH 4.7. The solution was then transferred into a separating funnel and shaken with 0.5 mL chloroform by vigorous shaking. The chloroform was collected in a 10 mL measuring cylinder and diluted to 5 mL with chloroform. The absorbance for the sample and standard solution was measured using UV-Vis spectrophotometer (Shimadzu, Japan) at 470 nm. The result was expressed as mg atropine equivalent per gram of extract (mg AE/g extract).

Cell culture conditions

Human colorectal adenocarcinoma (HT-29) and human cervical cancer (HeLa) were used as cancer cell models, while human embryonic kidney cells (HEK293) and immortalized human keratinocytes (HaCaT) were used as control normal cell lines. All cells were obtained from Indonesia International Institute for Life Sciences, Indonesia. The cells were cultured in flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). Both cells were placed in an incubator (CO_2 Incubator INC0108med, Memmert, Germany) at 37°C with 5% CO_2 for 24 – 48 h until the cells were reaching 90% confluency. The cells were harvested by trypsinizing the cells and obtaining the cell suspension.

Anti-proliferative study of P. neesii extract

The cell viability assay was employed to investigate the antiproliferative capability of *P. neesii* extract towards the cell models. The cells were seeded in a 96-well plate at a density of 5000 cells for HEK, HeLa, and HaCat cells and 7500 cells/well for HT-29 cells due to difference in growth rate. For 48 h, the cells were cultured in an incubator at 37°C and 5% CO_2. After 24 h, the cells were treated. The treatment groups were divided into: 12.5 to 100 µg/mL of *P. neesii* extract, negative control with no treatment, and 200 - 400 µM of 5-fluorouracil (5-FU) as positive control.[19] After 48 h, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was done to measure the cell viability. Briefly, 100 µL of media and 15 µL of MTS reagent (Promega, US) were added to each well after washing. The cells were then incubated for 3 hours in the dark. The absorbance was measured using a plate reader (The Infinite®M200 NanoQuant, TECAN, Switzerland) at 490 nm and the formula below was used to calculate cell viability:

$$Cell\ viability\ (\%) = \frac{control - blank}{sample - blank} \times 100\% \qquad \textbf{Eq. (1)}$$

Table 1. Collection results on the natural compounds of *Pogonatum* sp. (Accessed 6 July 2022).

Compounds	Classification	CID	Canonical SMILES	PubChem link
Sinapic Acid	Phenolic acid	637775	COC1=CC(=CC(=C1O)OC)C=CC(=O)O	https://pubchem.ncbi.nlm.nih.gov/compound/637775
Hydroxycinnamic Acid	Phenolic acid	637542	C1=CC(=CC=C1C=CC(=O)O)O	https://pubchem.ncbi.nlm.nih.gov/compound/637542
Vanillate	Phenolic acid	54675858	COC1=C(C=CC(=C1)C(=O)O)[O-]	https://pubchem.ncbi.nlm.nih.gov/compound/54675858
Gallate	Polyphenol	370	C1=C(C=C(C(=C1O)O)O)C(=O)O	https://pubchem.ncbi.nlm.nih.gov/compound/370
Chlorogenate	Polyphenol	1794427	C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)O	https://pubchem.ncbi.nlm.nih.gov/compound/1794427
Ferulate	Phenolic acid	445858	COC1=C(C=CC(=C1)C=CC(=O)O)O	https://pubchem.ncbi.nlm.nih.gov/compound/445858
Phloroglucinol	Phenol	359	C1=C(C=C(C=C1O)O)O	https://pubchem.ncbi.nlm.nih.gov/compound/359
Catechol	Polyphenol	289	C1=CC=C(C(=C1)O)O	https://pubchem.ncbi.nlm.nih.gov/compound/289

Results

In silico studies

Collection of natural compounds from P. neesi and target proteins

Secondary metabolites from *Pogonatum* sp and target protein were collected through the PubChem database as shown in Table 1. The natural compounds sample was collected through CID or ID compounds in PubChem and SMILES structure of each compound. Two target proteins were obtained from the PDB website, which included colon cancer (4LXD), and cervix cancer (4WXX), with protein resolutions of 1.90 Å and 2.62 Å respectively.

Biological activity analysis

The biological activities being analyzed in this study were anticarcinogenic and apoptosis agonist activity. The analysis was in the form of Pa score and Pi score from each natural compound, as recorded in Table 2. From these results it can be seen that all of the compounds possess a higher Pa score compared to Pi score. This shows that all of the studied compounds have the potential to act as an anticancer agent. Anticarcinogenic is the capability of the compound in inhibiting the growth of cancer cells, while apoptosis agonist is cell death caused by molecular mechanisms to eliminate damaged or infected cells.[20]

Toxicity analysis

From the results recorded in Table 3, it was shown that vanillate and phloroglucinol did not show activity against all four parameters. In addition, compounds with a low level of toxicity were 4-hydroxycinnamic acid and chlorogenate, with an LD_{50} of respectively 2850 mg/kg and 5000 mg/kg. Analysis was conducted to determine the toxicity class, LD_{50}, hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity from each natural compound.

Table 2. The biological analysis results of the natural compounds of *Pogonatum neesii.*

Compounds	Activity	Pa score	Pi score
Sinapic acid	Anticarcinogenic	0.616	0.012
	Apoptosis Agonist	0.661	0.019
Hydroxycinnamic acid	Anticarcinogenic	0.559	0.015
	Apoptosis Agonist	0.661	0.019
Vanillate	Anticarcinogenic	0.337	0.045
	Apoptosis Agonist	0.39	0.078
Gallate	Anticarcinogenic	0.395	0.031
	Apoptosis Agonist	0.562	0.031
Chlorogenate	Anticarcinogenic	0.846	0.004
	Apoptosis Agonist	0.589	0.028
Ferulate	Anticarcinogenic	0.616	0.012
	Apoptosis Agonist	0.702	0.015
Phloroglucinol	Anticarcinogenic	0.38	0.034
	Apoptosis Agonist	0.689	0.016
Catechol	Anticarcinogenic	0.357	0.039
	Apoptosis Agonist	0.647	0.021

Pa= Probability of activity; Pi= Probability of inactivity

Drug likeness analysis

Drug likeness analysis was based on two rules, Lipinski's and Veber's. The analysis for the Lipinski's rule of five was conducted against several parameters: Molecular weight should be less or equal to 500 Da, hydrogen bond donor (HBD) should not be more than 5, hydrogen bond acceptor (HBA) should not be more than 10, and the value of MlogP should be less or equal to 4.15.[21] Meanwhile, based on Veber's rule, rotatable bond (RB) parameters should not be more than 10 and the value of total polar surface area (TPSA) should be less or equal to 140 A.[22] The results presented in Table 4 showed that there was one violation against Lipinski's rule and one violation against Veber's rule. While all the other compounds fulfilled the criteria of drug feasibility.

Table 3. Results of toxicity analysis of the natural compounds of *Pogonatum neesii*.

Compounds	Toxicity class	LD_{50} (mg/kg)	Accuracy (%)	Hepatotoxicity	Immunotoxicity	Carcinogenicity	Mutagenicity	Cytotoxicity
Sinapic Acid	4	1772	70.97	0.54 (inactive)	0.89 (active)	0.67 (inactive)	0.87 (inactive)	0.96 (inactive)
Hydroxycinnamic Acid	5	2850	100	0.51 (inactive)	0.91 (inactive)	0.50 (active)	0.93 (inactive)	0.81 (inactive)
Vanillate	4	2000	69.26	0.52 (inactive)	0.99 (inactive)	0.70 (inactive)	0.95 (inactive)	0.93 (inactive)
Gallate	4	2000	70.97	0.61 (inactive)	0.99 (inactive)	0.56 (active)	0.94 (inactive)	0.91 (inactive)
Chlorogenate	5	5000	69.26	0.72 (inactive)	0.99 (active)	0.68 (inactive)	0.93 (inactive)	0.80 (inactive)
Ferulate	4	1772	70.97	0.51 (inactive)	0.91 (active)	0.61 (inactive)	0.96 (inactive)	0.88 (inactive)
Phloroglucinol	3	200	100	0.78 (inactive)	0.99 (inactive)	0.73 (inactive)	0.97 (inactive)	0.95 (inactive)
Catechol	3	100	100	0.82 (inactive)	0.99 (inactive)	0.84 (active)	0.9 (inactive)	0.87 (inactive)

Class I: fatal if swallowed ($LD_{50} \leq 5$), Class II: fatal if swallowed ($5 < LD_{50} \leq 50$), Class III: toxic if swallowed ($50 < LD_{50} \leq 300$), Class IV: harmful if swallowed ($300 < LD_{50} \leq 2000$), Class V: may be harmful if swallowed ($2000 < LD_{50} \leq 5000$), Class VI: non-toxic ($LD_{50} > 5000$).

Table 4. Results of drug feasibility analysis of the natural compounds of *Pogonatum neesii*.

Compounds	Lipinski's Rule				Veber's Rule		Lipinski's Violation	Veber's Violation
	MW (≤ 500 Da)	HBD (≤5)	HBA(≤10)	$_{M}\log_{P}$(≤4.15)	RB (≤10)	TPSA (<140 A)		
Sinapic Acid	224.21	2	5	0.73	4	75.99	0	0
Hydroxycinnamic Acid	164.16	2	3	1.28	2	57.53	0	0
Vanillate	167.14	1	4	0.74	2	69.59	0	0
Gallate	170.12	4	5	-0.16	1	97.99	0	0
Chlorogenate	354.31	6	9	-1.05	5	164.75	1	1
Ferulate	194.18	2	4	1	3	66.76	0	0
Phloroglucinol	126.11	3	3	0.18	0	60.69	0	0
Catechol	110.11	2	2	0.79	0	40.46	0	0

Molecular docking and visualization

Molecular docking was done to evaluate the binding affinity of the natural compound in *P. neesii* against each target protein. Table 5 records the binding affinity of the compounds on colon and cervical cancers. This analysis employed several reference compounds that generally interact with the target protein. Based on molecular docking it could be seen that the binding affinity value of the natural ligand in the protein was lower compared to the binding affinity value of the natural compound from *P. neesii*. Meanwhile, chlorogenate has the lowest binding affinity compared to other natural compounds in *P. neesii*.

Visualization of the molecular docking results was done to observe the structure and interaction between them. The interactions observed included the total number of interactions and the type of interaction. Supplementary Tables S1 and S2 (Supplementary Data) explain the types of interactions between the natural compounds on colon cancer

and cervical cancer target proteins respectively. Meanwhile, Figures 1 and 2 illustrate the 2D binding diagram and 3D conformation visualizations of the best molecular docking results, which was the visualization of chlorogenate on each of the target proteins in colon and cervical cancers respectively. As the compound with the lowest binding affinity of both target proteins, chlorogenate produced some of the highest number of bindings from all of eight compounds, with 16 bonds to 4LXD and 21 to 4WXX, second to vanillate with 25 bonds to 4WXX. Interestingly, most of the bonds were in the form of strong conventional hydrogen bonds with some weaker Van Der Waals bonds, such as the bonds to GLU1266, ARG1310, THR1525, THR1526, THR1528, GLN1536 to 4WXX protein and the bonds to THR5, GLN187, GLU88, ASP193, and GLU11 in 4LXD protein.

Extract characterization

P. neesii was characterized by TPC and TAC. Standard curves were constructed using the respective standards. *P. neesii* was shown to contain 48.654 ± 1.239 GAE mg/g of extract through TPC using the Folin-Ciocalteu method, and 25.356 ± 11.336 AE mg/g of extract through TAC assay.

Antiproliferative assay

MTS assay was used to determine the cytotoxicity of *P.neesii* extract towards various cell lines, including HEK293, HT-29, HeLa, and HaCaT cells, and the results were illustrated in Figure 3.

As shown in Figure 3a and 3b, both cancer cell lines HT-29 and HeLa cells that were treated with various concentrations of 5-FU showed a very significant decline in cell viability, whereas the treatment of *P. neesii* extract

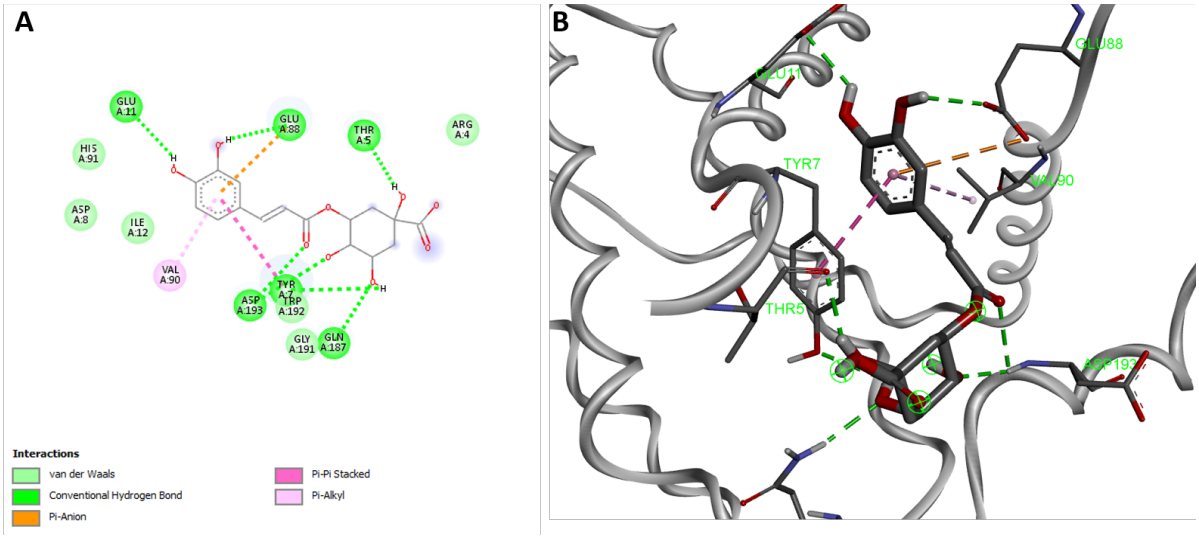

Figure 1. (a) Two-dimensional binding diagram and (b) three-dimensional visualization of chlorogenate on the target protein (PDB ID:4LXD) of colon cancer

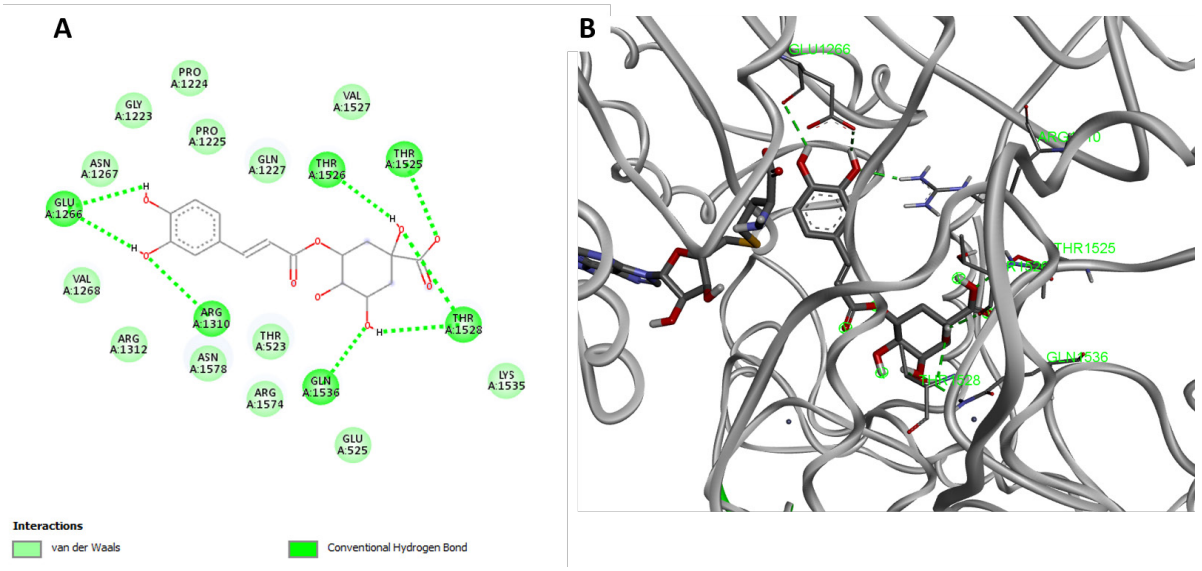

Figure 2. (a) Two-dimensional binding diagram and (b) three-dimensional visualization of chlorogenate on the target protein (PDB ID:4WXX) of cervical cancer.

Table 5. Molecular docking result on colon and cervical cancer target proteins.

Protein	Compound	Binding affinity	Protein	Compound	Binding affinity
	Navitoclax	-10.5		S-Adenosyl-L-Homocysteine	-8.5
	Chlorogenate	-6.9		Chlorogenate	-7.2
	Ferulate	-6.4		Ferulate	-6.8
	Hydroxicinnamic Acid	-6.1		Gallate	-6.3
DMT1 (4LXD)	Sinapic Acid	-5.8	**Bcl-2 (4WXX)**	Sinapic Acid	-5.8
	Gallate	-5.4		Vanillate	-5.7
	Vanillate	-5.4		Hydroxicinnamic Acid	-5.7
	Phloroglucinol	-4.8		Catechol	-5.2
	Catechol	-4.7		Phloroglucinol	-5.1

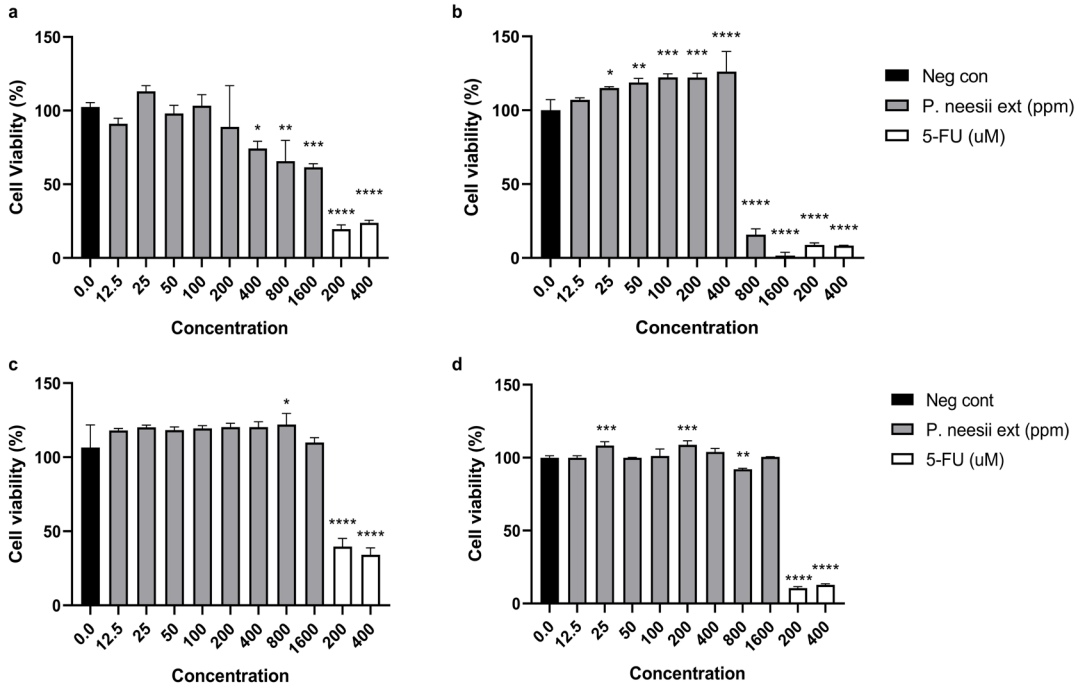

Figure 3. Cytotoxicity of P.neesii extract (ppm) and 5-fluorouracil (5-FU) (µM) on different cell lines (a) HT-29, (b) HeLa, (c) HaCaT, (d) HEK-293. The results were compared to negative controls where the cells were not treated with the extract or positive control (5-FU fluorouracil), and expressed as mean ± standard deviation of triplicates sample. One-way ANOVA followed with Dunnett's post hoc analysis were done on each group in respect to negative control. * Indicate statistical difference (P<0.05). ** Indicate statistical significant difference (P<0.002). *** Indicate statistical significant difference (P<0.0007) **** Indicate statistical significant difference (P<0.0001).

showed gradually decreasing cell viability, in which the extract exerted significant cytotoxic effects starting from 400 ppm and 800 ppm in HT-29 and HeLa cells respectively. Interestingly, the 5-FU also showed cytotoxic activity at the same concentrations on healthy cells, in which *P. neesii* extract did not show any cytotoxic effects at all at the same range of concentrations (Figure 3c and 3d).

Discussion

P. neesii is a species of bryophytes abundantly found in Indonesia, specifically in Cibodas Botanical Garden, Indonesia. In order to analyze the biological activity, analysis of the secondary metabolites found in *P. neesii* was conducted. Through *in silico* studies, vanillate and phloroglucinol did not show any potential toxicity on all tested parameters. In addition, based on the toxicity level, 4-hydroxycinnamic acid and chlorogenate showed the lowest toxicity at an LD_{50} of 2850 mg/kg and 5000 mg/kg respectively.

Based on molecular docking, chlorogenate has the lowest binding affinity value compared to the other natural compounds in *P. neesii*, even though the natural ligand from both proteins still has a higher binding affinity. Yet interestingly, the biological, drug feasibility, and toxicity analysis showed that the compounds of *Pogonatum sp.* should provide a good drug potency. The anticarcinogenic activity and considerably low toxicity should be a warrant for the next step of analysis. Molecular docking does provide a strong support for drug discovery, such as to help rationalizing ligands activity to a target of interest, virtual screening, to predict adverse drug reactions, and many more.[23] However, it still has limitations, such as the lack of confidence to give accurate binding energies and binding mode prediction due to the complexity of the

molecules[24], which usually stems from the conformational search algorithm and scoring function.[25] Thus, making this method usually combined with other methods[26], which is the purpose of the *in vitro* assessment in this study.

Moreover, a previous study stated that chlorogenic acid or chlorogenate is a highly potential molecule in inhibiting or interacting with the target proteins, which interacts with the active side of matrix metalloproteinases (MMP) through hydrogen bond.[27] The compound also has been extensively studied for anticancer properties by decreasing the number of the cancer cells[28], differentiation-inducing,[29] restricting cell cycle, inducing apoptosis, and suppressing the proliferation.[30–32] Even a chlorogenate-conjugated peptide also exhibits binding to breast tumor cell receptors.[33] DNMT1 is the most abundant human methyltransferase and plays an important role in the maintenance of 5'—C—phosphate—G—3' (CpG) DNA methylation patterns in the genome. The overexpression of this protein has been attributed to several cancer types, especially in colorectal cancer.[34] The hyperactivity of this protein in the colorectal cancer cells was observed compared to healthy cells using electrochemical platform.[35] This suggests that inhibiting the hyperactivity could be a strategy to treat colorectal cancer and other cancer related to DNMT1 expression and activity.[11] On the other hand, Bcl-2 protein is one of the popular targets for drug discovery in cancer. This anti apoptosis protein is expressed in several types of cancer, upregulated by estrogen in breast cancer, and supporting cancer cell to metastase, suggesting that inhibiting this protein could help overcome the cancer.[36-38]

The eight secondary metabolites found in *P. neesii* are classified as phenolic compounds. Therefore, in order to characterize the amounts of secondary metabolites in the extract, total phenolic, and alkaloid contents were measured. The TPC and TAC results showed that the extract did contain specific amounts of phenolic and alkaloid compounds. Phenolic compound is an important secondary metabolite and is a potential source of natural bioactive compounds which is often used commercially in various pharmaceuticals.[39] Ultimately, these compounds contribute to anticancer activities through the cell proliferation inhibition, cell-cycle arrest, apoptosis induction, MMP downregulation, Bcl-2 decrease, and many more.[4] In addition to that, studies also showed that the addition of phenolic acids with standard treatments could sensitize the drug-resistant cells, one of which is by decreasing the expression of multidrug resistance proteins, thus selectivity towards multidrug-resistant cancer cell types.[40]

Subsequent to measuring the secondary metabolite content, which showed the presence of secondary metabolites in the extract, as well as *in silico* studies, which showed the positive interaction of compounds (especially chlorogenate) in the extract against target proteins in cancer cells, *in vitro* studies were performed to analyze the actual selective cytotoxic effects of the extracts. Cancer cell lines in *in vitro* models that are widely used in medical research, especially in pharmaceutical development and basic cancer research.[41] HT-29 was used as the cancerous cell line in this study, as it has been widely employed in human colon cancer studies, where it has been proven to be comparable to *in vivo* models.[42] On the other hand, HeLa cells, the first human cells grown in culture that were isolated from a cervical carcinoma patient, have been widely used in cancer research.[43] HEK293 and HaCaT were used to represent normal cell lines to observe the cytoselectivity of *P. neesii* extract. Furthermore, 5-fluorouracil (5-FU), a chemotherapy agent, was used as a positive control as it has been extensively used for cancer treatments for decades. 5-FU acts by converting to fluorodeoxyuridine monophosphate, which creates a complex with thymidylate synthase which suppresses the formation of deoxythymidine monophosphate, which is required for replication reparation of DNA and whose deficiency causes cytotoxicity.[9] 5-FU showed cytotoxicity in both cancerous and normal cell lines. Chemotherapy drugs target rapidly dividing cells to induce DNA damage, which may also put healthy rapidly dividing cell lines at risk. Rapidly dividing cells are sensitive to chemotherapy as they increase the likelihood of chemotherapy drugs to interact with and disrupt their nuclear DNA.[44] This may explain the loss of cell viability of HaCaT cell lines treated with 5-FU. Hence, the non-specific protective mechanism of 5-FU may serve as a mediator for its toxicity towards healthy cells.[45]

According to the results shown in Figure 3, our *in vitro* experiment showed that *P. neesii* extract exerted very significant cytotoxic effects on the cancer cell lines, starting from 400 ppm on HT-29 and 800 ppm on HeLa cells, but did not exert cytotoxic effects at all on the normal cell lines, HaCaT and HEK293 cells. Interestingly enough, 5-FU showed cytotoxic effects on all cell lines, whereas the extract even increased the cell viability of HaCaT and HEK293 cells in some concentrations. This indicates that *P. neesii* extract showed a better cytoselective property than 5-FU, in which it exerted cytotoxic effects on cancer cell lines but not on normal cell lines.

Considering the result of *in silico* study found that the compounds of *P. neesii* still had lower binding affinity compared to the target's protein natural ligand, this may suggest that the compound in *P. neesii* could work in synergistic manner with each other and produce the cytotoxic effect observed in the *in vitro* part of this study as the extract were used in the crude manner, without any further purification after the first extraction. Future study should analyze the synergistic effect or antagonistic effect of the *P. neesi* crude extract.

Conclusion

Pogonatum neesii Dozy has been shown to have the potential as an anticancer agent through natural compounds that it contains and how those compounds bond with target proteins. The natural compound of *P. neesii* that has the lowest binding affinity is chlorogenate,

which means chlorogenate has the highest affinity towards target protein. In addition, analysis of the toxicity of chlorogenate is *toxicity class V* and has an LD_{50} of 5000 mg/kg, but based on the data there is 1 violation on Lipinski's rule and 1 violation on Veber's rule which can have an effect on the ADME drug parameters. Upon observing *in vitro* cell viability, the extract showed significant cytotoxic effects on cancerous cell lines, HeLa and HT-29 cells, but no cytotoxic effects on normal cell lines, HaCaT and HEK-293 cells, which showed the cytoselective potential of *P. neesii* extract as anticancer agent.

Author Contributions

Rizky Nurdiansyah: Conceptualization, Investigation, Formal Analysis, Resources, Funding, Writing - Review & Editing. Agus Budiawan Naro Putra: Conceptualization, Funding, Writing - Review & Editing. Ainun Nadhifah: Conceptualization, Funding, Writing - Review & Editing. Erika Chriscensia: Methodology, Investigation, Formal Analysis, Writing - Original Draft. Ulung Khoe Gondo Kusumo: Conceptualization, Investigation, Formal Analysis, Writing - Review & Editing. Stephanie Angela Yosiano: Conceptualization, Investigation, Formal Analysis, Writing - Original Draf. Anastasia Beatrix Musung: Conceptualization, Investigation, Formal Analysis, Writing - Original Draft. Sintikhe A Wenas: Conceptualization, Investigation, Formal Analysis, Writing - Original Draft. Steve Makalew: Conceptualization, Investigation, Formal Analysis, Writing - Original Draft Patricia Lovina: Conceptualization, Investigation, Formal Analysis, Writing - Original Draft. Intani Quarta Lailaty. Conceptualization, Investigation, Formal Analysis, Writing - Original Draft. Fransisca Aurelia Rahmad: Conceptualization, Investigation, Formal Analysis, Writing - Original Draft. Fandi Sutanto: Funding, Writing - Original Draft. Pietradewi Hartrianti: Conceptualization, Investigation, Formal Analysis, Resources, Funding, Writing - Review & Editing.

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Conflict of Interest

The authors report no conflicts of interest.

Supplementary Data

Supplementary data, Tables S1 and S2, are available at https://doi.org/10.34172/PS.2023.11.

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