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Research Article

Isolation of Bioactive Compounds from Two Orchid Species and Preliminary Test of Their Cytotoxicity Against T47D Breast Cancer Cells

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ABSTRACT

Research on plant natural products for screening of compounds with medicinal properties is mainly grow from the awareness on the use of herbal medicines and the side effects of most modern drugs. The potential of orchids in traditional medication has a long history, as indicated by ancient records on the use of many orchid species as medicinal plants. The objective of this study was identifying potential group of compounds with cytotoxic properties from orchid against T47D breast cancer cells. Two orchid species were used in this study, *Dendrobium lasianthera* and *Arachnis flos-aeris*. Chloroform, methanol, and aqueous extracts obtained from leaves, stem, and roots were tested for their cytotoxicity against T47D breast cancer cells to determine the most effective extract and fraction potential as anticancer. Determination on cytotoxicity was carried out using MTT assay, and cytotoxic property was indicated by IC_{50} value with doxorubicine as a positive control. Serial fractionation processes using vacuum liquid chromatography was done to find the most effective group of compounds demonstrating cytotoxicity. The result show that stem of *Dendrobium lasianthera* chloroform extract had higher cytotoxic activity than other extracts. The IC_{50} value of the most toxic combining fraction was 122,66 µg/ml. The result of the separation and purification truly increase the cytotoxicity effect of isolate against T47D breast cancer cells, proven by the IC_{50} value of middle isolate was 37,11 µg/ml while other isolates were higher than those of doxorubicine (IC_{50} = 61 µg/ml). TLC analysis showed that the most active compound obtained was terpenoid and phenolic.

Keywords: orchids, cytotoxicity, breast cancer, medicinal plant

INTRODUCTION

Orchids belong to the family of Orchidaceae, the second largest family of flowering plants in the world in terms of its of species. Apart from a vast number of genus recognized as ornamental plants, one of the most widely known members of orchid family is *Vanilla* which is used as food flavoring. The potential of orchids in medicine has a long history, as indicated ancient records on the use of orchids as medicinal plants use were noted mainly by Chinese, Sumerian, Indian, and Egyptian[1]. In Indonesia, literature records indicated orchids as group of plants with particular importance was dated back on 18th century with Rumphius's famous work on "Herbarium Amboinense", and on 19th century by the work of Boorsma on alkaloids extracted from orchids from Bogor Botanical Garden[2]. The emergence of scientific research on orchid as medicinal plant was dated back on 1935 when infusion from dried stem of *Dendrobium moniliformae* was reported to exhibit antipyretic and analgesic properties[3]. During 1990s research on anticancer from natural product were numerous, and 61% of anticancer agents are natural products or their derivates[2]. Denbinobin and moscatilin, two compounds isolated from the orchid genus *Dendrobium*, were widely explored for their bioactivity as anticancer. Denbinobin, a naturally occurring phenanthroquinone isolated from the genus *Dendrobium*, is known to have anticancer activity by inducing apoptosis in cancer cells, such as on pancreatic adenocarcinoma cell line[4]. Denbinobin isolated from *D. nobile* was reported to be cytotoxic against A549 (human lung carcinoma), SK-OV-3 (human ovary adenocarcinoma), and HL-60 (human promyelocytic leukaemia) cell lines[3]. Plant extracts of *D. nobile* also has been noted as having antitumor activity against Dalton's Lymphoma Ascites cell lines[5]. Meanwhile, moscatilin isolated from *D. loddigesii* was reported to induce apoptosis on human colorectal cancer cells[6]. In current study chloroform, methanol, and aqueous extracts from leaves, stem, and root of *Dendrobium lasianthera*, and *Arachnis flos-aeris*, were tested for their cytotoxic properties against T47D breast cancer cells. These two orchid species were chosen due to the availability as well as being familiar plants for people in Indonesia, especially Yogyakarta. Determination on cytotoxicity of these plant extracts was carried out using MTT assay. The IC_{50} value was used as indication of cytotoxicity to determine the most effective extract and

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fraction having potential as anticancer. Serial fractionation processes using vacuum liquid chromatography was conducted to obtain the most effective compound demonstrating cytotoxicity against T47D breast cancer cells.

MATERIALS AND METHODS

Materials

Plant materials used in this study were two orchids species, *Dendrobium lasianthera*, and *Arachnis flos-aeris*, purchased from a nursery in Sleman, Indonesia. Plant identification was done at the Plant Systematics Laboratory, Faculty of Biology Universitas Gadjah Mada. The T47D breast cancer cell line was obtained from Parasitology Department, Faculty of Medicine Universitas Gadjah Mada. Chemicals for cell culture and anti-proliferative test were: DMEM culture medium, penicillin-streptomycin, FBS 10% v/v, PBS 1x, Trypsin 0.25%, MTT, stopper reagent SDS 10% in 1N of HCl, DMSO, and ethanol 70%. Chemicals for extraction, fractionation, and chromatography of plant compounds were consisted of chloroform, ethyl acetate, methanol, n-hexane, acetone, glacial acetic acid, petroleum ether (p.a. grade), distilled water, silica gel plate GF_{254}, spray reagent cerium (IV) sulphate, liebeman-burchard, vanillin sulphate, dragendorff, and ammonium.

Methods

Plant compounds extraction

Procedure for the extraction of plant compounds using chloroform and methanol was performed as followed: air dried leaves, stems, and roots were ground using mortar and pestle. An amount of 150 g of grounded plant materials were macerated three times until the solvent come into clear liquid. Each maceration process was done for 24 hours. The extracts were filtered and air dried. The dried filtrates were than store in the glass tubes. Extraction of plant compounds using water as the most polar solvent is conducted by grinding plant materials, which then 150 g of the grounded material was boiled in 250 ml of water for 30 minutes. The resulted filtrate was then saturated using water bath evaporator.

MTT assay for the plant extracts

Cytotoxicity test of plant extracts against T47D breast cancer cells using MTT assay was conducted following a previous procedure[7]. A volume of 100 µl suspension of T47D cell line culture containing 1.5 x 10^4 cells was filled in each well of 96-wells microplates. Plant extracts with a volume ranging from 50 to 400 µg/ml, with the interval of 50 µg/ml, as cytotoxicity treatments were added to the cell culture with four replicates for each treatment. The treated cell culture is then incubated for 24 hours on 37°C, 5% CO_2. At the end of incubation time, the culture media was discarded, and the cells were washed up using PBS. A volume of 110 µl MTT solution was then added into each well, followed by incubating the cells on 37°C and 5% CO_2 for 4 hours. Upon completion of incubation, 100 µl of stopper reagent SDS 10% was added to dilute the purple formazan crystals. The cell absorbance was read using ELISA reader on λ=595 nm.

Thin Layer Chromatography (TLC) of plant extracts

This step was carried out to get the most suitable eluent for fractionation process. A series of mixture of solvent was used, those ranging from the lowest to the highest polarity. The stationary phase used was silica gel GF_{254}.

Fractionation of potential plant extracts using Vacuum Liquid Chromatography

Fractionation of potential plant extracts was conducted according to standard procedure[8]. The stationary phase used in this process is silica gel GF_{254}, and the mobile phase was a series of solvent with various polarities as those used in TLC. Fractionation process was run by slowly pouring the solvent onto filter paper placed on top of the silica gel, and onto the plant extracts placed on the sintered glass, followed by vacuuming process. The solution resulted from this process was collected in a porcelain container. After being fully air-dried, then weight of each fraction was calculated.

MTT assay for the fractions

The cytotoxicity of potential fractions against T47D cancer cells was carried out using a standard procedure[7] as described on the previous section.

Preparative Thin Layer Chromatography (PTLC) of potential fractions

This step was performed to further purification of potent fraction derived from VLC fractionation using PTLC procedure to obtain fractions or bioactive compounds with higher purity level. The stationary phase used in this procedure was silica gel $GF_{254.}$ The combined fractions showing the highest potential in cytotoxicity were spotted onto PTLC plate in a horizontal sequence. The rest of the process was the same as those for TLC until the plate was fully air-dried. The resulted bands on the plate were divided into sections based on the number of spots, followed by manually scrapping the bands using spatula to get a powder. The resulted powder from each band was diluted with the same solvents used in PTLC, and then run through a sintered glass for filtration process. The filtrates were then air-dried and weighted.

MTT assay for the PLTC isolates

The cytotoxicity of PLTC isolates against T47D cancer cells was carried out using a standard procedure[7] as described on the previous section.

Identification of bioactive compounds using TLC and compared with standard compounds

Identification of bioactive compounds demonstrated the highest cytotoxicity against T47D breast cancer cell line was carried out using TLC, sprayed with various reagents and visualized using UV light with λ=254 nm and 366 nm. Compounds identification was done by comparing the spots developed on TLC plate with standard compounds.

RESULTS AND DISCUSSION

The extraction method used in this study was maceration, which was suitable for isolation of various compounds including those which have low temperature evaporation point. This method was considered to have advantageous properties and high recovery compared to other extraction methods, especially for crude extract from plant materials[9, 10]. The recovery of extract from three different plant organs isolated using various solvent varied considerably (Table

Table 1. Recovery of extract from leaves, stems, and roots using various solvent

Orchid species	Plant organs	Sample weight (g)	Recovery (g)		
			$CHCL_3$	Methanol	Water
Arachnis flos-aeris	Root	50	0.81	1.42	1.61
	Stem	50	0.39	0.47	0.96
	Leaf	20	0.73	1.05	6.01
Dendrobium lasianthera	Root	50	0.84	0.66	0.59
	Stem	20	0.96	0.67	2.38
	Leaf	20	0.84	1.33	5.12

Table 2. IC_{50} value of various extracts isolated from three different plant organs

Orchids species	Vegetative organs	IC_{50} values (µg/ml)		
		Chloroform	Methanol	Water
Arachnis flos-aeris	Root	186	457	727
	Stem	139	590	356
	Leaf	272	1368	1436
Dendrobium lasianthera	Root	140	471	628
	Stem	117	390	457
	Leaf	358	542	396
Doxorubicin		61		

Table 3. Recovery and solvent composition of each fraction

Fraction number	Solvent	Recovery (g)
Fraction 1	n-hexane	0.05
Fraction 2	n-hexane	0.04
Fraction 3	chloroform 100%	1.31
Fraction 4	chloroform 100%	0.20
Fraction 5	chloroform:ethyl acetate 40:1	0.13
Fraction 6	chloroform: ethyl acetate 40:1	0.09
Fraction 7	chloroform: ethyl acetate 30:1	0.08
Fraction 8	chloroform: ethyl acetate 30:1	0.06
Fraction 9	chloroform: ethyl acetate 20:1	0.05
Fraction 10	chloroform: ethyl acetate 20:1	0.02
Fraction 11	chloroform: ethyl acetate 10:1	0.03
Fraction 12	chloroform: ethyl acetate 10:1	0.03
Fraction 13	chloroform: ethyl acetate 1:1	0.12
Fraction 14	chloroform: ethyl acetate 1:1	0.06
Fraction 15	Etil asetat 100%	0.07
Fraction 16	Etil asetat 100%	0.04
Fraction 17	Metanol 100%	0.24
Fraction 18	Metanol 100%	0.03
Fraction 19	Kloroform:metanol 1:1	0.05
Fraction 20	Kloroform:metanol 1:1	0.03

1). The highest recovery in material extraction was found in the leaf water extract (5.12 – 6.09 grams from 20 grams sample). This indicated that water could extract the highest numbers or concentration of compounds in the leaf compared to other solvents in other plant organs. The use of water used for the extraction of bioactive compound from plant materials were reported in previous studies[11,12]. Results of cytotoxicity test of plant extracts on T47D breast cancer cells were indicated by IC_{50} values, as shown in Table 2. All six extracts with three different solvents demonstrated cytotoxic activity onT47D breast cancer cells higher than the positive control, doxorubicine (61 µg/ml), a common anticancer medicine. The most toxic one was the chloroform extract of *D. lasianthera* stem with the IC_{50} value of 117 µg/ml. Based on this result, only the chloroform extract of *D.lasianthera* stem was chosen for further analysis. In the TLC analysis a series of solvent was used, ranging from the lowest to the highest polarity. The chromatogram profiles were visualized using visible light, UV light with λ=254 nm and 366 nm. Results of TLC analysis indicated that the most suitable eluent was a mixture of chloroform : ethyl acetate = 10:1. Fractionation step was done to separate the compounds in the chloroform extract to get particular fractions containing less number of compounds, which facilitated further identification[13]. Results of fractionation on the most potent extract using vacuum column chromatography were showed on Table 3. From the 20 fractions obtained in previous steps, those showing similarities were combined for further analysis. Monitoring the composition of compounds was done by

Table 4. Combined fractions and recovery

Number of fraction	Combined fraction	Recovery (g)
1 and 2	A	0.09
3	B	1.31
4,5 and 6	C	0.44
7,8,9,10,11 and 12	D	0.16
13,14 and 15	E	0.20
16,17, and 18	F	0.28
19 and 20	G	0.03

Table 5. The IC_{50} of combined fractions from the most potential extracts

Combined fraction	IC_{50} values (µg/ml)
A	199.00
B	205.71
C	122.66
D	133.57
E	161.37
F	513.38
G	821.25

Table 6: The Recovery of isolate from the most toxic fraction

Combined isolates	Recovery (g)
Upper isolate	0.03
Middle isolate	0.03
Lower isolate	0.02

Table 7. IC_{50} of isolates from the most toxic combining fraction.

Combined isolates	IC_{50} values (µg/ml)
Upper isolate	1436.25
Middle isolate	37.11
Lower isolate	70.50

running the extract on the TLC plate using suitable eluent. The fractions which had similar profiles were combined together, which resulted in the recognition of 7 groups of fractions coded as A to G (Table 4). Detail of the combined fractions with their respective recovery was listed in Table 4. Furthermore, the compound compositions of combined fractions were examined using TLC, with the results shown in Figure 1. Subsequent cytotoxicity test was performed using the combined fractions. The IC_{50} value showed that the most potent combined fraction was C with IC_{50} value of 122,66 µg/ml (Table 5). Cytotoxicity of the most toxic combined fraction again T47D breast cancer cells was higher than those of doxorubicin, and results showed the increase of orchids extract toxicity compered to crude extracts. Therefore, the subsequent step was performed by simplifying the fraction and reducing the number of compounds of combined fraction (C) using Preparative-TLC. Preparative-TLC was applied in this step because this method was suitable for compound separation, simple, cheap, and show the ability to separate small amount of compound[16, 17]. By several development processes, it was found that the combination of n-hexan : ethyl acetate : methanol = 4:1:1 was the best eluent for the

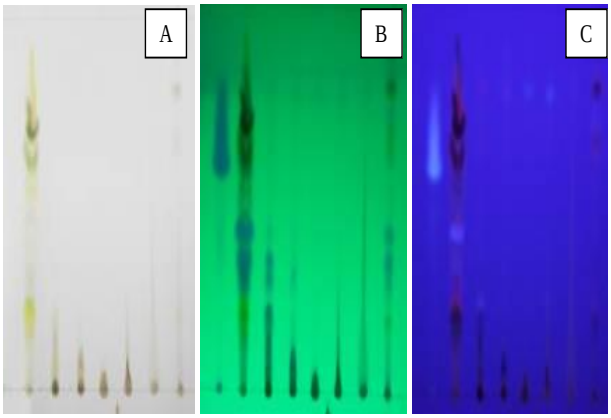

Figure 1. TLC profiles of seven combined fractions starting from combined fraction A from the left to G at the right. (A). visible light; (B). UV light 254 nm; (C). UV light 366 nm.

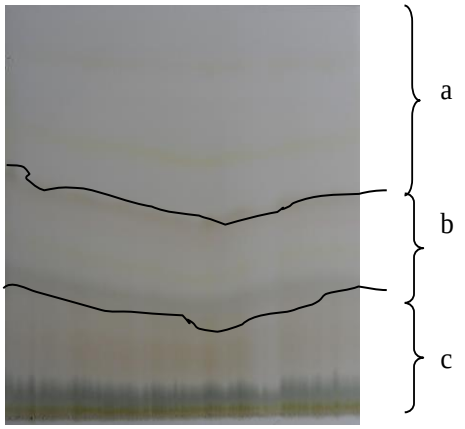

Figure 2. Profiles PTLC of the most potent combining fraction (with eluen n-hexan : ethyl acetate : methanol = 4:1:1). a. upper fraction; b. medium fraction; c. lower fraction

running of fraction C on P-TLC. Monitoring active compounds in the most toxic combined fraction was carried out using color reaction on TLC plate using serium sulfate sprayer and visualized under UV light with wavelength λ=254 nm and 366 nm. The TLC development showed that there were 3 groups of spot (Figure 2.). The following step was marking the area, scratched and collected three different spots in three different glass tubes. The collected materials were extracted with chloroform : etyl asetate = 1:1 using magnetic stirrer for 15 minutes. The recovery was shown in Table 6. The three different extracts were then tested for their cytotoxicity again T47D breast cancer cells. Results showed that the separation and purification activity increased the cytotoxicity effect of isolate against T47D breast cancer cells, as proven by the IC_{50} value of middle isolate. The value was 37,11 µg/ml (Tabel 7) while other isolates were higher than those of doxorubicin. Based on color reaction on TLC plate of middle isolate, it was recognized that the isolate was consisted of several kind of compounds. Cytotoxicity test result of combined fraction showed that extract purification increased the cytotoxicity effect on T47D breast cancer cells compared to those of crude extracts.

Table 6. Compound groups identified from the most toxic fraction

Sprayer compounds	Identified group of compounds	Color	Result
Serium (IV) sulfat	Terpenoid	Purple spot, brown	positive
Lieberman-Burchard	Triterpenoid	Dark spot, blue	negative
Vanilin sulfat	Terpenoid	Purple spot, brown	positive
Sitroborat	Flavonoid	Yellow spot	negative
Dragendorf	Alkaloid	Orange spot	negative
$AlCl_3$	Phenolic	Yellow spot	positive

The increase in cytotoxicity effect was due to the antagonism effect of active compounds in the combined fraction. The increases in synergism effect of active compound occur due to the increase in speed reaction and bioavailability of active compound[14, 15]. Nevertheless, the antagonism effect of active compounds will reduce speed reaction and bioavailability of active compound. After identifying the most potential of cytotoxic agent, the following step was detection of compound groups of the respective fraction using spraying detection reagent on TLC. Based on the color reaction, the most toxic fraction was recognized as consisted of several groups of compound identified as terpenoid and phenolic compounds (Table 6). Terpenoid and phenolic compounds have been explored for their cytotoxic properties, and hence considered as potential anti-cancer natural products. The potential of terpenoid as group of compounds with cytotoxic properties has been recognized such as those extracted from *Curcuma* rhizome[18]. Meanwhile, the potential of phenolic compounds in cytotoxicity test against T47D cancer cells has been reported from extracts of *Garcinia cowa*[19].

Results of this study, therefore, showed that chloroform extract of *D. lasianthera* stem which demonstrated cytotoxic against T47D cancer cells could be considered as a potential anti-cancer natural product. Identification of the fraction showing highest cytotoxicity revealed that terpenoid and phenolic compounds were detected in this potential herbal extract.

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