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CAFFEINE ISOLATION AND CYTOTOXIC ACTIVITY OF CHLOROFORM FRACTION FROM ETHANOL EXTRACT OF DURIOKUTEJENSIS AGAINST T47D CELL LINE

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ABSTRACT

Background and Aims: Approximately 19 of 28 species of durian were grown in Borneo. One of them, Duriokutejensis (*durian pulu*) have not been explored for its potential in anti-cancer treatments. This study aimed to isolate chemical compounds and to evaluate the cytotoxicity of the crude extract and isolated compounds from the chloroform fraction of durian pulu's stem bark against breast cancer T47D cell line.

Methods: The extracts were screened for phytochemicals such as flavonoid, terpenoid, alkaloid, saponin, tannin, polyphenol, and anthraquinone. The vacuum liquid chromatography (VLC) technique was employed to extract fractionation. Isolation and purification were performed using Sephadex column chromatography. The structure of the pure compound was elucidated by spectroscopic analysis (1H NMR, 13C NMR, COSY, HMBC, and HMQC). The extract and isolated compound were tested against T47D by MTT assay. One compound, caffeine, was isolated from the chloroform fraction.

Result: The IC50 values of ethanol extract and caffeine of the stem bark were 331.82 and 361.59 µg/mL, respectively.

Conclusion: The findings showed that both the crude extract and its isolated compound have no potential of cytotoxic activity against breast cancer T47D cell line.

Keywords: Breast Cancer, Caffeine, Cytotoxic, DurioKutejensis, Phytochemical, T47D Cell Line

1. INTRODUCTION

According to Rokom (2018), breast cancer has the highest incidence in Indonesia compared to other kinds of cancer. Breast cancer is one of the leading causes of death among women in the world (Siegel, Miller, &Jemal, 2018). In cancer therapy, using cytotoxic treatments could create problems by their side effect or their susceptibility to drug resistance (Rachmani, Suhesti, &Widiastuti, 2012).

Kalimantan Island has an ecosystem that allows various plants to grow. One genus of plants that widely found in Borneo is Durio, approximately 19 of 28 species of Durio grow on the island. Previous studies on the extract of Duriozibethinus revealed its medicinal properties, such as anti-hypercholesterolemic, antiatherosclerosis, antimicrobial, and antidiabetic activity (Lim, 2013; Muhtadi, Primarianti, &Sujono, 2015; Muhtadi, Haryoto, Sujono, &Suhendi, 2013). A phytochemical study reported flavonoid, steroid, and glycoside content in the ethanol extract of Duriozibethinus, which has an antidiabetic property (Aruan, Barus, Haro, Siburian, &Simanjuntak, 2019). Interestingly, a bitter taste in Duriozibethinus was related to caffeine according to one study (Voon et al., 2007). Besides, Arung et al. (2015) reported the fruit extract of Duriokutejensis has an antioxidant effect by suppressing ROS formation, while Chingsuwanrote et al., (2016) showed the decrease of tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) secretion. Previous research found several secondary metabolites such as 3β-O-trans-caffeoyl-2α-hydroxyolean-12-en-28-oic acid, 3β-O-trans-caffeoyl-2α-hydroxytaraxest-12-en-28-oic acid, maslinic acid, arjunolic acid, 2,6-dimethoxy-p-benzoquinone, and fraxidin in the stem part of Duriokutejensis (Lim, 2013). Boehmenan in the ethanolic extract of Durioaffinis stem bark exhibited cytotoxicity activity against T47D cell line (IC50 = 13.7 µg/mL) (Rudiyansyahet al., 2014). Concerning to the breast cancer treatment, this study investigated the cytotoxicity of the crude extract and the isolated compound from the chloroform fraction of Duriokutejensis stem bark ethanol extract against breast cancer T47D cell line to explore the potential compounds in anticancer treatment.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

The chemicals and materials used in this study were analysis-grade chloroform (99% purity), ethyl acetate, and technical-grade n-hexane to extract Duriokutejensis stem bark, T47D cell line, DMSO, the reagent for MTT [(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)], phosphate buffer saline (PBS), Roswell Park Memorial Institute (RPMI) Medium, trypsin-EDTA 0.25%, sodium dodecyl sulfate (SDS) 10%, gelatin, and FeCl3, and reagents for phytochemical screening. This study used an inverted microscope (Olympus CKX41), CO2 incubator (Nuaire TM IR auto flow), an ELISA reader, a Laminar Air Flow (Labconco), a hemocytometer, a cell counter, and a vortex (Genie). All reagents and chemicals were purchased from Sigma chemicals and Merck chemicals.

2.2 Preparation of extract and isolation

The fresh stem bark of Duriokutejensis was collected from Palangkaraya, Central Borneo, Indonesia, and identified at the Biological Research Center, SebelasMaret University, Solo with the plant determination report number 209/UN27.96.4/Lab/2017. A voucher specimen was prepared and deposited at the Faculty of Pharmacy, University of Muhammadiyah Surakarta, Indonesia. The stem bark was cleaned, cut and dried in a cabinet dryer at 40ºC. It was then ground into a fine powder and stored in airtight bottles. The dried powder was extracted by maceration in pure ethanol, with a ratio of extract to solvent by 1:10, at room temperature for 72 hours. This mixture was filtered and the filtrate was concentrated using a vacuum evaporator at 40-50°C. It yielded a dark brown residue (233.78 g, 7.8%). The fractionation of stem bark ethanol extract of Duriokutejensis was carried out by using VLC. Hexane, chloroform, and ethyl acetate were used as the mobile phase in different ratios of increasing polarity from hexane to ethyl acetate. The gradient concentration of the mobile phase was the combinations of CHCl3: hexane (9:1), CHCl3 (100%), CHCl3: ethyl acetate (9:1) and CHCl3: ethyl acetate (7:3). The isolation and purification were performed using Sephadex column chromatography with methanol as a solvent. The protocol resulted in 9 fractions (1 from ethanol and 8 from chloroform). The semi polar fraction from chloroform were fractionated in series and produced 3 fractions (2-3, 4, and 7-9). This study focused on the sub-fraction 2-3 of chloroform after the eluent using 1: 1: 1 of methanol: acetonitrile: water. The sub fraction was a white odorless powder (15.7 mg) and called compound 1. The extraction protocol was inspired by Contini et al., (2008) method.

2.3 Phytochemical screening

The extracts were screened for phytochemicals such as flavonoid, terpenoid, alkaloid, and saponin. Flavonoid was tested for by using the Bate-Smith and Metcalf methods. A 37% concentration of hydrochloric acid (HCl) solution was employed as the test solution. The solution was heated in a water bath and observed to form a color. A bright red color indicating the presence of flavonoids (Lestari, Himawan, Abadi, &Retnowati, 2015). The Salkowski test was used to detect terpenoid. The crude extract was dissolved in chloroform and it was added to the concentrated Sulphur acid (H2SO4). If a reddish-brown color was formed, terpenoid presence is confirmed (Lestari et al., 2015). The presence of alkaloids was screened for by Mayer's test. The extract was added to HCl (2N), heated in a water bath, and then cooled before adding NaCl. The mixture was stirred and filtered and the obtained filtrate was combined with HCl (2N) and Mayer's reagent. The white precipitation indicated alkaloid content (Lestari et al., 2015). The foam test was used to confirm the presence of saponin. The test solution was added to distilled water and shaken vigorously. The stable persistent froth for 20 minutes indicated the presence of saponin (Zohra et al., 2012). The tests for tannin, polyphenol, and anthraquinone constituents followed using the previously described methods (Doctor et al., 2014). The

Liebermann-Burchard test was used for unsaturated sterols. The tests for tannins and polyphenols were carried out by subjecting the plant extracts in Gelatin test and Ferric chloride test. The presence of anthraquinones was also tested employing both the Borntrager's and Modified Borntrager's tests. As much as 5 grams of extract was added to 10 ml of benzene, filtered, and added with ammonia solution (Doctor et al., 2014).

2.4 In-Vitro Cytotoxicity Assay

T47D human breast cancer cell line from the Microbiology lab of Pharmacy Department of University Muhammadiyah Surakarta was used to do in-vitro cytotoxicity assay. The cell line was cultured in an RPMI medium for 48 hours before the assay. A cell suspension in a 100 µL RPMI culture medium (density of 1.5 x 104 cells/wells) was inserted into a 96-well plate and then incubated at 37 ˚C with 5% CO2 for 24 hours. The assay was performed in triplicate. There were four assessed variables, cells control (RPMI media with T47D cells), media control (RPMI media only), treatment groups (each extract, non-polar, semi-polar, and polar fractions with the concentration of 5, 25, 50, 100, 500, and 1000 µg/mL per well), and positive control (doxorubicin at concentration 6.25, 12.5, 25, 50, and 100 µg/mL). As much as 100µL of each different concentrations of those four were added to the T47D cells, placed in a 96-well plate, and incubated at 37º C for 24 hours. At the end of the treatment, 100 µL of MTT was added to each well and the microtiter plates were incubated for 24 hours at 37º C. As much as 100 µL of SDS was then added to each well and the plate was incubated for another 24 hours at room temperature to dissolve the formazan, which was the product of the reaction between mitochondrial enzymes of living cells with MTT. At the end of the incubation period, the light absorbance was recorded with an ELISA microplate reader at a wavelength of 594 nm (Fotakis&Timbrell, 2006; van et al., 2011; Cancer Chemoprevention Research Center (CCRC), 2019).

2.5 Data Analysis

Structure elucidation of compound 1 was based on 1D and 2D NMR spectral analyses including 1H, 13C-NMR, COSY, HMQC, and HMBC. The percentage of the living cells from cytotoxic activity result was calculated based on the equation:% living cells = (treatment absorbance - absorbance media control)/(absorbance of control cells -absorbance media control) x 100 %.

Linear regression of log concentration vs % living cells is used Y = BX + A, where Y is a probit number and X is log concentration. Probit value of 50% living cells was inserted into the equation to obtain the IC50 values (Suhendi et al., 2014).

3. RESULT AND DISCUSSION

3.1 Extract and isolation

The isolation on the ethanol extract of Duriokutejensis stem bark has led to a pure compound 1. It was obtained from the chloroform fraction of the third group in subfraction 2-3 (5 SF 2-3), as a white odorless powder.

The 13C-NMR spectrum shows eight spectra at 140-150 ppm, C-6 (δC 155,53), C-2 (δC 151,82), C-4 (δC 148,77), C-5 (δC 107,70), and C-8 (δC 141,50), in addition to three methyl at δC 33,71 (N7-CH3), δC 29,86 (N3-CH3), and δC 29,44 (N1-CH3). The 1H-NMR spectrum shows one proton at δH 7.5 ppm, and three methyl singles at δH 3.39 (N1-CH3), δH 3.46 (N3-CH3), and δH 3.57 (N7-CH3) ppm. Based on 2D NMR, 13C-NMR and 1H-NMR data analysis and literature data (Verma& Kumar, 2010; Yang et al., 2012), compound 1 was identified as caffeine (Table 1).

Table 1. ^{13}C NMR and ^{1}H NMR of caffeine.

No	Isolate Sub Fraction 2-3		Caffeine reference (Verma & Kumar, 2010)	
	δ ^{13}C-NMR	δ ^{1}H-NMR	δ ^{13}C-NMR	δ ^{1}H-NMR
2	151.82	-	151.52	-
4	148.77	-	148.53	-
5	107.70	-	107.39	-
6	155.53	-	155.21	-
8	141.50	7.50 (s)	141.28	7.51 (s)
N1-CH_3	29.44	3.39 (s)	29.54	3.59 (s)
N3-CH_3	29.86	3.46 (s)	27.72	3.41(s)
N7-CH_3	33.71	3.57 (s)	33.39	4.0 s)

3.2 Phytochemical screening

Based on the results of phytochemical screening, the ethanol extract had the presence of flavonoid, terpenoid, tannin, and polyphenol compounds (Table 2). The phenolic compound in the flavonoid could be responsible for the cytotoxic activity with many mechanisms of action, such as carcinogen inactivation, cell cycle arrest, and inhibition of angiogenesis, a reversal of

multidrug resistance, antiproliferation, induction of apoptosis and differentiation, antioxidation or a combination of these mechanisms (Ren, Qiao, Wang, Zhu, & Zhang, 2003).

Table 2. Phytochemical screening of Duriokutejensis' stem bark ethanolic extract.

No	Phytochemical Screening	Result	Method
1	Flavonoid	Positive (+)	Bate-Smith & Metcalf
2	Terpenoid	Positive (+)	Salkowski Test
3	Alkaloid	Negative (-)	Mayer's Test
4	Saponin	Negative (-)	Foam Test
5	Tannin and Polyphenol	Positive (+)	Gelatin and $FeCl_3$ Test
6	Anthraquinone	Negative (-)	Brontager's Test

3.3 In-vitro cytotoxicity assay

The cytotoxic assay is a qualitative and quantitative test to determine the cell death process. The control cell is indicated by dark purple while the positive control is indicated by light purple (Figure 2). It indicated that the normal cells have reduced the tetrazolium salt into the purple crystal of formazan, while the doxorubicin treatment did not show similar results, indicating that the cell has died.

There are not many study about the cytotoxicity of Durio genus. A previous study found the cytotoxicity effect of ethanol extract and boehmenan isolate compound of Durioaffinis to T47D using MTT assay with the IC50 value of 828.3 and 13.7 µg/mL, respectively (Rudiyansyah et al. 2014). The IC50 values from our Duriokutejensis ethanol extract (331,82 µg/mL) and Durioaffinis (828,3 µg/mL) are categorized as inactive as the value exceed 100 µg/mL. Meanwhile, the Durioaffinis (boehmenan) is the active cytotoxic agent to T47D (IC50 of 13,7 µg/mL).

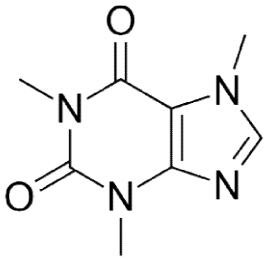

Figure 1. CaffeineSkeletal formula of caffeine. Created with ChemDoodle and Adobe Illustrator CC 2017

For a compound to exhibit positive cytotoxic activity, it should meet the following criteria: IC50 value ≤ 20 µg / mL as active, IC50 value 10-100 µg / mL as moderate, IC50 value >100 µg / mL as has no cytotoxic activity (Jabit et al., 2009; Kuete et al., 2013; Sajjadi et al., 2015). The cytotoxic effect of the ethanol extract in comparison with doxorubicin on T47D cell line is determined by MTT (Table 3). The study showed that the ethanol extract and the resulting compound 1 have no cytotoxic activity with IC50 values of 331,82 and 361,59 µg/mL respectively, with doxorubicin at 11,49 µg/mL. Compound 1, known as caffeine, shows no anticancer activity. However, it has been suggested by previous studies that caffeine can increase antitumor activity if given in conjunction with cisplatin in treating hepatocellular carcinoma (HCC) (Kawano et al., 2012). As such, this study warrants further study on the activity of the obtained isolates to determine their ability to overcome the T47D cell line.

Table 3. The cytotoxicity of the *DurioKutejensis'* stem bark ethanolic extract against T47D cell lines.

Parameter	IC_{50} (µg/mL)
Ethanol Extract	331,82
Caffeine	361,59
Positive Control: Doxorubicin	11,49

4. CONCLUSION

Based on the findings, it is concluded that there are flavonoids, terpenoid, tannin, and polyphenol compounds in the stem bark of DurioKutejensis. One compound was confirmed as caffeine. The evaluation of cytotoxic activity showed that the ethanol extract has a greater potential of cytotoxic activity against T47D cell line compared to caffeine. However, both of them have no cytotoxic activity against T47D cell line.

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