RESEARCH ARTICLE

Cytotoxic Effect and Constituent Profile of Alkaloid Fractions from Ethanolic Extract of *Ficus septica* Burm. f. Leaves on T47D Breast Cancer Cells

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Abstract

The study aimed to investigate the profile of alkaloids in two ethyl acetate soluble fractions, namely fractions A and B from an ethanolic extract of *Ficus septica* leaves and cytotoxic effect on T47D breast cancer cells. Preparation of both fractions involved maceration of leaves with 70% (v/v) ethanol, filtration with Al_2O_3, precipitation with 0.1 N HCl, Mayer reagent, and 0.1 N NaOH, and also partition with ethyl acetate. Qualitative thin layer chromatography (TLC) was conducted to determine the profile of alkaloids in the two fractions, using alkaloid specific reagents such as Dragendorff, sodium nitrite, and Van Urk-Salkowski. Cytotoxic effects of both fractions on T47D cells were evaluated using MTT assay with a concentration series of 1.56; 3.12; 6.25; 12.5; 25 and 50 μg/mL. The TLC test showed that fractions A and B contained alkaloids with Rx values of 0.74 and 0.80 for fraction A and 0.74, 0.84, 0.92 for fraction B with regard to yohimbine using the mobile phase of n-buthanol:glacial acetic acid:distilled water (3:1:1 v/v/v). Moreover, an indole alkaloid was detected with Rx values of 0.80 and 0.84, respectively. Fractions A and B exhibited high cytotoxic effects on T47D cells with IC50 values of 2.57 and 2.73 μg/mL, respectively. In conclusion, overall the results of this study showed that fractions of *Ficus septica* contain alkaloids including indole alkaloid or its derivatives and possess a cytotoxic effect on T47D cells. This research supports the idea that alkaloids in *F. septica* have anticancer activity.

Keywords: *Ficus septica* Burm. f. - T47D breast cancer cells - cytotoxic activity - alkaloids

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Introduction

Cancer is a disease associated with uncontrolled growth and spread of abnormal cells (American Cancer Society, 2014). The abnormal cells are characterized with an alteration in cell differentiation and cell communication with its extracellular environment (Pusztai et al., 1995). With its characteristics, cancer has become one of the leading causes of morbidity and mortality in the world. According to a World Health Organization (WHO) global report, number of new cancer cases reached 14.1 million, and 8.2 million for cancer-related deaths in 2012 (Ferlay et al., 2013). Therefore, looking at this high numbers, numerous attempts have been done in order to discover new treatment for cancer. The attempts include studies on chemotherapy agents from medicinal plants.

Previous study reported that alkaloid compounds in Awar-awar (Ficus septica) are potential as a cytotoxic agent (Wu et al., 2002; Lansky et al., 2008). Ethanolic extract of *Ficus septica* Burm. f. leaves exhibited a cytotoxic effect on human T47D breast cancer cell line with IC_{50} value of 13.0 μg/mL (Pratama, 2010). The ethanolic extract of *F. septica* leaves was gradually fractionated to provide some fractions. Among the fractions, n-hexane insoluble fraction and ethyl acetate soluble fraction exhibited most potent cytotoxic effect on T47D cell line with IC_{50} value of 9.3 and 13.7 μg/mL, respectively (Nugroho et al., 2011a). These fractions exhibited synergistic effects with doxorubicin on T47D cell line (Nugroho et al., 2012a; Nugroho et al., 2013a). The fraction also exhibited immunomodulatory effect in the in vitro and in vivo studies (Nugroho et al., 2012b; Nastiti et al., 2014).

Perceiving alkaloid profile of *F. septica* leaves is advantageous in chemotherapy agent, and anticancer drug discovery for it may serve as a leading compound. To date, various studies in regard to cytotoxic effect of *F. septica* leaves have not investigated the profile of its alkaloid compounds, despite its cytotoxic effect has been recognized (Wu et al., 2002). Based on previous positive results on ethyl acetate soluble fraction, thus the present study investigates the profile of alkaloids in the ethyl acetate fraction with water-soluble components (fraction A) and ethyl acetate fraction with ethanol-soluble

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components (fraction B) from ethanolic extract of *F. septica* leaves and their cytotoxic effect on human T47D breast cancer cell line.

Materials and Methods

Materials

F. septica leaves were collected from Sumber Arum, Moyudan, Yogyakarta Indonesia on July 2013 and were identified at Laboratory of Pharmacognosy, Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The voucher specimen was deposited in herbarium of the department. Materials for alkaloids profile determination were yohimbine hydrochloride (catalog number Y3125) (Sigma-Aldrich, Germany), and silica gel 60 F_{254} Thin Layer Chromatography (TLC) plate (Merck, Germany). Mayer reagent, Dragendorff spray reagent, sodium nitrite ($NaNO_2$) spray reagent, and Van Urk-Salkowski spray reagent were obtained from the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. Materials for assay were dimethyl sulfoxide (DMSO) (Gibco Invitrogen, USA), doxorubicin (Kalbe Farma, Indonesia), and [3 - (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] (MTT) (Bio Basic, Canada). The final DMSO concentration was 1%. Doxorubicin was used for in vitro experiment by diluting the stock solution to provide the final desired concentration. Microplate reader (Bio-Rad microplate reader Benchmark serial no. 11565) was used to measure the absorbance at 595 nm for the MTT colorimetric reaction in the cytotoxicity assay.

Preparation of extract

Dried leaves of *F. septica* were grinded to form a coarse powder (500 g). The coarse powder was macerated with total of 5 L 70% ethanol (1:10 w/v). Firstly, powder was macerated with 2.5 L (1:5 w/v) ethanol for 24 hours. Subsequently, the filtrate was collected, while the residue was re-macerated. Re-maceration of residue used 70% ethanol with a ratio of 1:2.5 w/v for 24 hours. The re-maceration was done twice. All filtrates were collected and evaporated for 12 hours to form a viscous ethanolic extract with a weight of 40 g. To provide extract's fractions, the viscous ethanolic extract was diluted with hot distilled water. The distilled water have pH value of 2-3 after addition with 0.1 N HCl. Secondly, the diluted extract was filtrated through a column packed with 83.12 g of aluminium (III) oxide (Al_2O_3) (1:2 w/w). Filtration was replicated twice. Each replication used new Al_2O_3 powder with the same weight. Finally, filtrate was collected and evaporated on a waterbath yielding filtrate A. Whilst, unfiltrated residue was collected and extracted with 96% ethanol. Filtrate was collected and evaporated on a waterbath yielding filtrate B.

Preparation of extract's fractions

Separately, filtrate A and filtrate B were added with 0.1 N HCl drops until a final pH ranging 2 to 3. Mayer reagent was added to each test solution until alkaloids precipitate is formed. Each precipitate was obtained after centrifugation. Its supernatant was removed while alkaloids precipitate remained on the bottom of each test tube. Each tube was thoroughly added with 0.1 N HCl to dissolve the precipitate. The tube was placed in an ultrasonicator bath to enhance the dissolving process. Each solution was added with 0.1 N NaOH to reach a final pH value of 8-9. Each solution was partitioned with ethyl acetate yielding ethyl acetate soluble fraction and insoluble fraction. Both ethyl acetate soluble fractions were recalled as fraction A and fraction B.

Determination of alkaloids profile

The determination of alkaloids profile was performed with a Thin Layer Chromatography (TLC) assay. As the stationary phase, silica gel 60 F_{254} TLC plates were used. Mobile phase was a solvent system of n-buthanol : acetate acid glacial: distilled water with a ratio of (3:1:1 v/v/v). Yohimbine hydrochloride (Sigma-Aldrich, Germany) was as used an alkaloid standard. A 10 μl each fraction (fraction A and B) was spotted on separated TLC plates as samples. A 4 μl of yohimbine was spotted on each plate as a standard of alkaloid. Following the separation processes, spraying agents were used on separated TLC plates to determine alkaloids profile. The spraying agents were Dragendorff, $NaNO_2$, and Van Urk-Salkowski. Alkaloids profile was determined based on Rx value. This value was measured using the following formula:

$$Rx = \frac{\text{distance traveled by sample from original line (cm)}}{\text{distance traveled by standard from original line (cm)}}$$

T47D cell culture

Human T47D breast cancer cell line was obtained from Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Indonesia and grown in Roswell Memorial Park Institute (RPMI) medium containing 10% fetal bovine serum (Gibco, USA), 2% penicillin-streptomycin (Gibco, USA), and fungizone 0.5% (Gibco, USA) in a flask in a humidified atmosphere (5% CO_2) at 37°C.

Cytotoxicity assay

T47D cells viability was assessed using a MTT (Bio Basic, Canada) colorimetric assay. The cells were cultured in 96-well plates and each well contained $5x10^4$ cells/100 μL. The culture cells were incubated in a humidified incubator at 37°C in an atmosphere of 5% CO_2 for 24 hours. Cell confluence or crowding of cells in the plate was 80%, counted with hemocytometer and cell counter. After 24 hours incubation, culture medium was discarded. The cells were treated by either fractions of *F. septica* or Doxorubicin (control group), and then incubated for 24 hours. The concentrations of the fractions were 1.56; 3.12; 6.25; 12.5; 25; 50 μg/mL in cell culture. After incubation, the cells were incubated with 0.5 mg/mL MTT for 4 hours in 37°C. Viable cells react with MTT to produce purple formazan crystals. After 3-4 hours, the 10% SDS stopper solution (Gibco, USA) in 0.1N HCl (Merck, Germany) was added to dissolve the formazan crystal. The cells were then incubated for 24 hours at room temperature and protected from light. After incubation, the 96-well plate

was shaken using a horizontal shaker for homogenization, and the absorbance was measured by a microplate reader (Bio-Rad, Tokyo, Japan) at 595 nm.

Data analysis

Experimental data from the cytotoxicity assay was MTT absorbance. Cell absorbance holds a positive correlation with the number of viable cells. Then, cell absorbance was converted to percentage of viable cells by this following equation:

$$\text{Cells viability (\%)} = \frac{(B-C)}{(A-C)} \times 100\%$$

Where A, B, and C stand for absorbance of control group, treatment group, and cell culture medium, respectively. The acquired data was plotted on a graph expressing the relation between concentrations of fractions and cell viability.

Potency of cytotoxic effect was represented by IC_{50} value. The value of IC_{50} expresses a concentration of the fractions which can give a reduction to cell viability by 50%. IC_{50} was calculated using probit analysis based on linear regression relationship between the logarithm of concentration of fraction (independent variable) and the percentage of cell viability (dependent variable).

Results

Fractions of F. septica leaves and their alkaloids profile

Two different fractions were provided according to the scheme in figure 1. Using the alkaloids TLC identification method from Spangenberg et al. (2008) with Dragendorff spray and sodium nitrite spray reagents, the alkaloids spots were well-visualized. Two consecutive sprays with Dragendorff and sodium nitrite reagents was able to enhance the visualization of the alkaloids spots for its discolored background. Prior to the sodium nitrite spray, the alkaloids spots were less visualized. The color of the spots was orange, similar to the background. The alkaloids spots seen after spraying are shown in Figure 2. There were six alkaloids spots, two spots of fraction A and four spots of fraction B.

The result of this TLC alkaloid identification was in alignment with prior alkaloids precipitation test. The alkaloids precipitation test was performed using Mayer and Dragendorff reagents and giving positive results. Both reagents were added to test solutions. Precipitate was formed on both test solutions from both reagents. Precipitate gave cream and reddish brown color with Mayer and Dragendorff reagents, respectively. The precipitate color indicated presence of alkaloids in both fractions (Harborne, 1973; Nelima et al., 2011). A further investigation on alkaloids profile was performed with a Van Urk-Salkowski test developed by Ehmann (1977). Figure 2 showed the visualized spots. There were two spots visualized, one for each fraction. We can conclude that both fractions contain alkaloids. Alkaloids profile is determined with Rx value in regard to yohimbine. The Rx values of the spots were 0.80 and 0.84 for fraction A and fraction B, respectively.

Effect of fractions of F. septica leaves on T47D cells viability

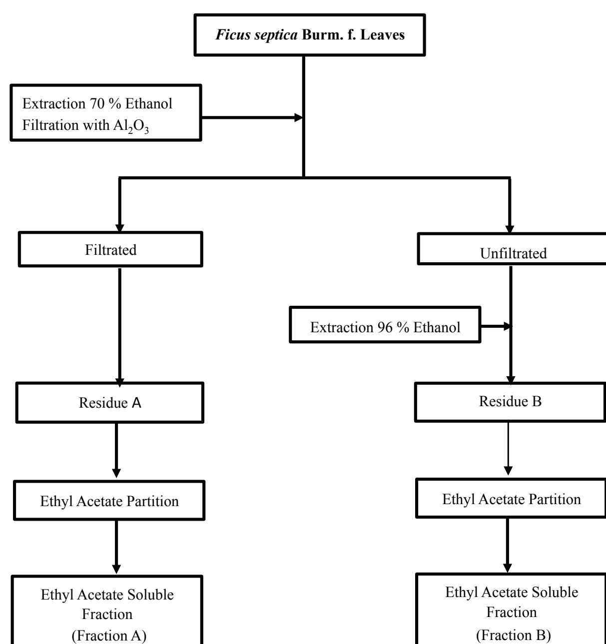

Figure 1. Scheme of Procedures for Obtaining Fraction A and Fraction B from Ficus Septica Burm. f. Leaves

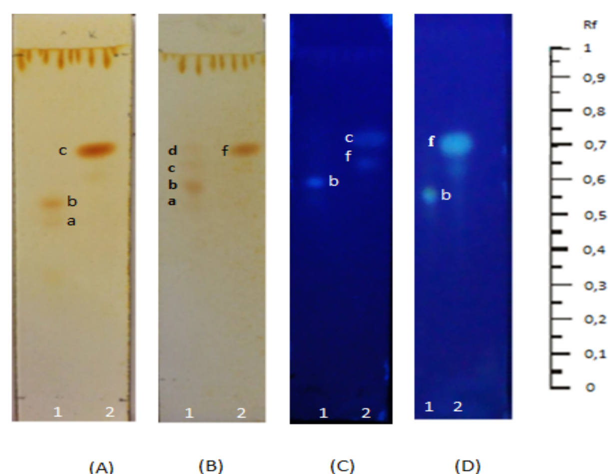

Figure 2. TLC profiles of Fraction A, Fraction B, and Standard using n-Buthanol: Glacial Acetic Acid: Distilled Water (3:1:1 v/v/v) as Mobile Phase after Dragendorff-sodium Nitrite Spraying and Van Urk-Salkowski Spraying. Plate (1): lane 1 is fraction A and lane 2 is standard. TLC plate was visualized after Dragendorff-sodium nitrite spraying under visible light. There were two spots found (a & b). These spots indicated presence of alkaloids. Its Rx values were 0.74 and 0.80 respectively. Plate (2): lane 1 is fraction B and lane 2 is standard. TLC plate was visualized after Dragendorff-sodium nitrite spraying under visible light. There were four spots found (a, b, c, d). These spots indicated presence of alkaloids. Its Rx values were 0.74; 0.84; 0.93; 1 respectively. Plate (3): lane 1 is fraction A and lane 2 is standard. TLC plate was visualized after Van Urk-Salkowski spraying under UV 366 nm light. There was one spot found (b). This spot indicated presence of indole alkaloids or its derivatives. Its Rx value was 0.80. Plate (4): lane 1 is fraction B and lane 2 is standard. TLC plate was visualized after Van Urk-Salkowski spraying under UV 366 nm light. There was one spot found (b). This spot indicated presence of indole

Concentration (μg/mL)	Fraction A	Fraction B
1.56	51.632±1.690	52.148±0.188
3.12	48.729±0.709	50.408±1.610
6.25	42.993±0.930	42.741±0.518
12.50	18.132±1.158	21.479±2.403
25	2.461±0.664	1.305±0.331
50	2.059±0.329	0.381±0.144

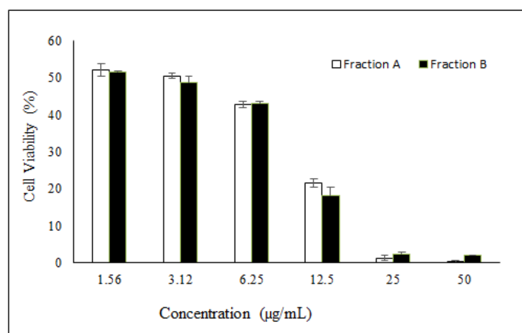

Figure 3. Effect of Fraction A and Fraction B on T47D Cells Viability using MTT Method. T47D cells were incubated with 1.56; 3.12; 6.25; 12.50; 25; 50 μg/mL of both Fraction A and B for 24 hours at 37oC. Data shown comes from two replication. Data are expressed as (mean±SEM).

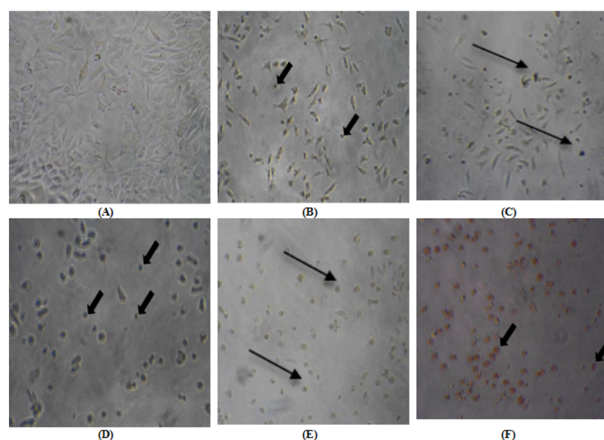

Figure 4. Effect of Fraction A and Fraction B on Cell Viability of T47D Cells Tested by MTT Method. Cell morphology was observed under microscope after incubation with (A) vehicle control; (B) Fraction A 12.5 μg/mL; (C) Fraction B 12.5 μg/mL; (D) Fraction A 50 μg/mL; (E) Fraction B 50 μg/mL; (F) Doxorubicin as positive control. Dead cells are pointed by black arrows

Figures 3-4 show the effect of fraction A and fraction B in their concentration series on T47D cells viability for 24 hours incubation. Fraction A and fraction B affected T47D cells viability by inhibiting cell growth and reducing the number of viable cells. In the study, fraction A could decrease the cell viability in concentration-dependent manner. Highest concentration of fraction A (50 μg/mL) could inhibit cell growth resulting in 2.059±0.329% cell viability. Concentrations of fraction B also decreased the cell viability in concentration-dependent manner. Fraction B on its highest concentration (50 μg/mL) could inhibit cell growth and left 0.381±.0.144% viable cells. IC_{50} values of fraction A and fraction B were 2.57 μg/mL and 2.73 μg/mL, respectively.

Changes in T47D morphology are shown on Figure 4. Viable cells have a morphology similar to epithelial cells, while dead cells have smaller size. Dead cells changes morphologically to a circular form. The results indicated that fraction A and fraction B showed high cytotoxicity effect on T47D breast cancer cell line.

Discussion

Biodiversity of Indonesia is second largest in the world after Brazil including medicinal plants. Exploration of the medicinal plants is one approach in the discovery and development of drugs. The exploration includes pharmacological activities, herbal formulation, phytochemical studies etc (Nugroho et al., 2011b; Nugroho et al., 2011c; Nugroho et al., 2013b; Nugroho et al., 2014a-c). Since the cancer is the biggest killer in Indonesia, the studies of medicinal plants for the treatment of this disease become interesting (Sarmoko et al., 2014; Setiawati et al., 2014).

In present study, both fractions obtained from *F. septica leaves* exhibited a cytotoxicity effect on T47D cells. Reportedly, some alkaloids of *F. septica* showed cytotoxicity effect on HCT-9 cell line, NUGC cell line, and HONE-1 cell line (Damu et al., 2009). Some alkaloids of *F. septica* exhibited anti-inflammatory activity through Cyclooxygenase-2 (COX-2) and Inducible Nitric Oxide Synthase (iNOS) enzyme inhibition (Yang et al., 2006). Cancer cell activity may involve COX-2 and iNOS, thus the alkaloids have potential in anticancer activity.

Inevitably, the cytotoxicity effect comes from its phytochemical compounds. Results of previous studies indicated that *F. septica* contains alkaloids (Damu et al., 2005; Vital et al., 2010). Several alkaloids classes have been identified and known belong to alkaloids classes of indolizidine, phenanthroindolizidine, pyrolidine, caprophenone, and indole (Hegnauer, 1969; Gellert, 1982; Ueda et al., 2009; Nastiti, 2013). Intensive studies need to be conducted in order to identify remaining alkaloids in *F. septica*.

A specific cellular target or mechanism of action of alkaloids in *F. septica* have not been identified clearly (Yang, et al., 2006; Chemler, 2009). Several known alkaloids have been studied including tylophorine, antofine, and tylocrebrine. To date, their known mechanism of action varies from protein, DNA, and RNA synthesis inhibition (Chemler, 2009).

Alkaloids profile determined in this study is important for future studies. The determined alkaloids profile by the Rx value enables us to set a criteria we would like to achieve in future studies. By having the Rx values of alkaloids, it would be easier for future studies to determine the alkaloids profile they get, whether it is the same as presented in this study or not. The Rx value may help in isolation of the alkaloids in future studies.

Our study counts heavily on extracting as many alkaloids as possible. It was achieved by the extraction method which used acid-base extraction and ethyl acetate partition. Acid-base extraction is known as a method to perceive alkaloids compounds from crude extracts (Siwon, 1982). The alkaloid-containing extract was mixed with acid solution to convert the alkaloids into their salt form. Alkaloid salts are soluble in a polar solvent, however, their

base form are soluble in a non-polar solvent. Filtration process through a column using Al_2O_3 functions to trap the inorganic ballast, phenolic compounds, some flavonoids etc (Haznagy-Radnai, 2007). Afterwards the tertiary amine alkaloids-containing fractions were dissolved in ethyl acetate. Several known alkaloids of *F. septica* are tertiary amine alkaloids. Thus, the ethyl acetate soluble fraction was used as the test solution in this study.

The present study has proved the acid-base extraction followed by ethyl acetate partition is an effective method to perceive alkaloids from *F. septica* with higher cytotoxicity effect on T47D cell lines in comparison to this of the fractions in previous studies. The fractions in this study exhibited lower valuese of IC_{50} which make them more potential to serve as an chemotherapeutic agent. We propose that the method and alkaloids profile in this study are important within the effort in discovering a chemotherapeutic agent from *F. septica* leaves. However, the molecular mechanism of the ethyl acetate soluble fraction still needs further investigations.

Ethyl acetate soluble fraction was yielded from acid-base extraction of ethanolic extract of *F. septica* leaves. The alkaloids-containing *F. septica* leaves exhibited a high cytotoxic effect on T47D cells. The fraction is potential to be developed as a chemotherapeutic agent in breast cancer therapy. Alkaloids profile contained in the fractions has been determined in the form of Rx value in regard to yohimbine. The data is crucial for further studies of extraction and isolation of alkaloids from *F. septica* leaves.

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100.0
75.0
50.0
25.0
0
6.3
56.3
31.3
Newly diagnosed without treatment