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Original Research Article

Cytotoxic Effect of Ethanolic Extract Fractions of Indonesia Plant Ficus septica Burm. F. on Human Breast Cancer T47D cell lines

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Abstract

Ficus septica Burm. F. (Moraceae) is a plant which grows widely in some areas of Indonesia and other Southeast Asia countries. Previously, ethanolic extract of the plant leaves exhibit cytotoxic effect on several cell lines including T47D cells. The extract also showed chemoprevention effect in vivo. In the study, the ethanolic extract was fractionated gradually by n-hexane and ethyl acetate to yield four fractions including n-hexane soluble fraction, n-hexane insoluble fraction, ethyl acetate soluble fraction and ethyl acetate insoluble fraction. These fractions were then investigated for their cytotoxic effect on T47D cells. The cell viability were assessed using MTT colorimetric assay. The results showed that the cytotoxic effect of n-hexane insoluble fraction (IC_{50} : 9.3 µg/mL) was much more potent than this of n-hexane soluble fraction (IC_{50} : 331.0 µg/mL). Hexane insoluble fraction was then fractionated with ethyl acetate to provide ethyl acetate soluble fraction and ethyl acetate insoluble fraction. The cytotoxic effect of ethyl acetate soluble fraction (IC_{50} : 13.7 µg/mL) was much more potent than this of ethyl acetate insoluble fraction (IC_{50}: >700 µg/mL). In conclusion, n-hexane insoluble fraction and ethyl acetate soluble fraction exhibited potent cytotoxic on breasr cancer T47D cell lines. The fractions are potential to be developed as anticancer agent in breast cancer therapy.

Keywords : Ficus septica Burm. F., cancer, T47D cells, cytotoxic.

Introduction

Cancer is a disease related to uncontrolled rapid growth of abnormal cells in the body. They can invade the healhty surrounding tissue and spread to other organs. This process is known as metastatis. Cancer is also named a malignant tumours and neoplasms [1-3]. Cancer is one of non-communicable diseases and a public health problem in the world. Cancer is a major cause of death in the world, around 13% of all deaths in 2008. More than 70% of all death due to cancer occured in developing countries. One of main types of cancer is breast cancer [3]. The incidence of breast cancer is increasing every year. Breast cancer is the leading cause of cancer death. In the United States, breast cancer is a type of cancer that occur most commonly in women (28% of total cases) [4]. In Indonesia, percentage incidence of breast cancer is the second highest rank after cervical cancer [5].

There are several approaches used for treating cancer such as surgical excision, irradiation and chemotherapy. The uses of these methods depend on the tumor type and the stage of cancer development [2]. Due to the increasing incidence

of breast cancer every year, the attempt to discover new anticancer agents is also increasing. Several study have been done to find out anticancer agents from natural products (medicinal plants) for preventing and treating breast cancer.

Indonesia is the second larger country in the world after Brazil in terms of biodiversity including medicinal plants. One of Indonesian medicinal plant is Awar-awar or Ficus septica Burm. F. (Moraceae). This plant originates from and grows widely in some areas of Indonesia and other Southeast Asia countries. Previously, our research group named Indonesian Cancer Chemoprevention Research Center (CCRC) has selected several Indonesian plants for their cytotoxic effect on breast cancer T47D cell lines. One of them with a potent cytotoxic effect was ethanolic extract of Ficus septica Burm. F. Treatment of the extract on breast cancer T47D cell lines for a period of 24 hours resuted in a cytotoxic effect with IC_{50} value of 13 µg/mL. The extract also showed synergistic effect in combination with chemotherapeutic agent doxorubicin on the concentration of 4.88 µg/mL (Ficus septica extract) and 3.75 nM (Doxorubicin) [6]. Another study, ethanolic extract of Ficus septica leaves induced apoptosis in breast cancer cells MCF-7. The extract also downregulated the expression of Bcl-2 protein [7]. In vivo study, chemopreventive effect of the ethanolic extract was studied in 7,12-dimethylbenz[a]nthracene (DMBA)-induced rat liver cancer. The extract (750 mg/kg BW) induced apoptosis throught p53-independent pathway in 7,12-dimethylbenz[a]nthracene-induced rat liver cancer [8].

In present study, we investigated the cytotoxic effect of fractions of ethanolic extract of Ficus septica Burm. F. leaves on breast cancer T47D cells line The ethanolic extract was prepared by macerating the dried-leaves powder with 70% ethanol. The extract was then fractionated using n-hexane yielding two fractions of hexane soluble fraction and insoluble fraction. The insoluble fraction of n-hexane was then fractionated using ethyl acetate yielding ethyl acetate soluble fraction and insoluble fraction. The cytotoxic activity of fractions were then compared using IC_{50} values, a potency parameter of cytotoxic effect.

Materials and Methods

Materials

Ficus septica Burm. F. was collected from area around Sumber Arum Moyudan, Yogyakarta, Indonesia. Ficus septica Burm. F. was identified by a botanist at Pharmaceutical Biology Department, Universitas Gadjah Mada, and the voucher specimen was deposited in herbarium of the department. Material for cytotoxic assay were [3 - (4,5-dimetilthiazol-2-yl) -2.5-diphenyl tetrazolium bromide] (MTT) (Sigma Chemical, St Loius, MO), H_2O_2 (Lab Vision Plus), chromogen 3,3-diaminobenzidin (DAB) (Novo Castra).

Preparation of Ficus septica ethanolic extract fractions.

In brief, dried ground powder of fresh leaves of Ficus septica Burm. F. was extracted using ethanol 70% with a ratio of 1:5 for 72 hours. Then, the filtrate obtained was filtered, while the sediment was re-extracted using ethanol 70% at a ratio of 1:2 for 72 hours. The re-extraction was done twice. The extract was then collected, and evaporated under reduced pressure to give of viscous ethanolic extract. The extract was added with 100 mL aquadest, and then mixed to yield liquid form of ethanolic extract. The extract was fractionated with n-hexane at a ratio of 140:400 (ethanolic extract:n-hexane) yielding two fractions of hexane soluble fraction and insoluble fraction. The insoluble fraction of n-hexane was then fractionated using ethyl acetate at a ratio of 140:250 (insoluble fraction of n-hexane: ethyl acetate) yielding ethyl acetate soluble fraction and insoluble fraction of ethyl acetate. Four fractions obtained were concentrated by rotary vacuum evaporator to obtain viscous extract. The fractions were dried using freeze drying to eliminate the existence of the remaining traces of water.

T47D Cell Culture.

Human breast carcinoma T47D was obtained from Prof. Masashi Kawaichi (Nara Institute Sciences and Technology, Japan). The cells were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% Fetal Bovine Serum (Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin 1% (Gibco), and fungizon 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO_2) at 37°C.

Cytotoxic Assay.

T47D cell viability were assessed using MTT colorimetric assay (3-[4,5-diethylthiazol-2-yl]-2,5-dipheniltetrazolium bromide (Sigma St. Louis, MO, U.S.A.). The cells were cultured in 96-well plates (Becton Dickinson Co., NJ, USA), and each well contained $5x10^3$ cells. The culture cells were incubated in a humidified incubator at 37°C in an atmosphere of 5% CO2 and 95% air for 24 hours. Cell confluence or crowding of **cells** in the plate was about 70-80%. After 24 hours incubation, culture medium was discarded. The cells were treated by either Ficus septica Burm. F. ethanolic extract fractions (treatment groups) or the vehicle (control group), and then incubated for 24 hours. The concentrations of the fraction were 1, 10, 50, 100, 250, 500 and 700 µg/mL in DMEM. 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 4 hours, the stopper 10 % SDS (Sigma Co., St.louis, MO) in 0.01 N HCl (Merck) was added to dissolve the formazan crystal. The cells were then incubated for 24 hours in room temperature and protected from light. After incubation, the cells were shaken, and cells absorbance was measured by ELISA reader at λ 595 nm.

Data Analysis

The experimental data was absorbance of each well, and then converted to percentage of viable cells as described below.

Percentage of viable cells = [B-C/A-C]x100%. A, B and C are absorbances of control group, treatment group and medium, respectively.

The potency of cytotoxic effect is represented by IC_{50} value calculated using probit analysis. IC_{50} value represents a concentration of the fractions that produce cells death of 50%. Calculation of IC_{50} values based on linear regression relationship between logarithm of concentration versus probit value of the percentage of cell viability.

Statistical analysis

All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test were used for statistical analyses. P-values less than 0.05 were considered significant.

Results

Previous study, ethanolic extract of Ficus septica Burm. F. leaves showed potent cytotoxic activity on T47D cell lines. Subsequently, the ethanolic extract were then fractionated gradually using n-hexane and ethyl acetate yielding four fractions of hexane soluble fraction, hexane insoluble fraction, ethyl acetate soluble fraction, and ethyl acetate insoluble fraction. The procedure to obtain these fractions described in Fig 1. In the study, we investigated these fractions on T47D cell lines. The potency of cytotoxic effects of the fractions were then compared using IC_{50} values.

Effect of n-hexane soluble fraction on T47D cell viability

Figures 2-3 show the effects of a series concentration of n-hexane soluble fraction of Ficus septica Burm. F. leaves on the viability of breast cancer T47D cell lines for 24 hours incubation. All concentrations used in the experiment could decrease the cells viability significantly ($P<0.05$) in concentration-dependent manner (Fig. 2). At highest concentration (700 µg/mL), the fraction decreased the cell viability by 59.33±2.41 %. IC_{50} value of n-hexane soluble fraction of Ficus septica Burm. F. leaves was 331.0 µg/mL (Table 1).

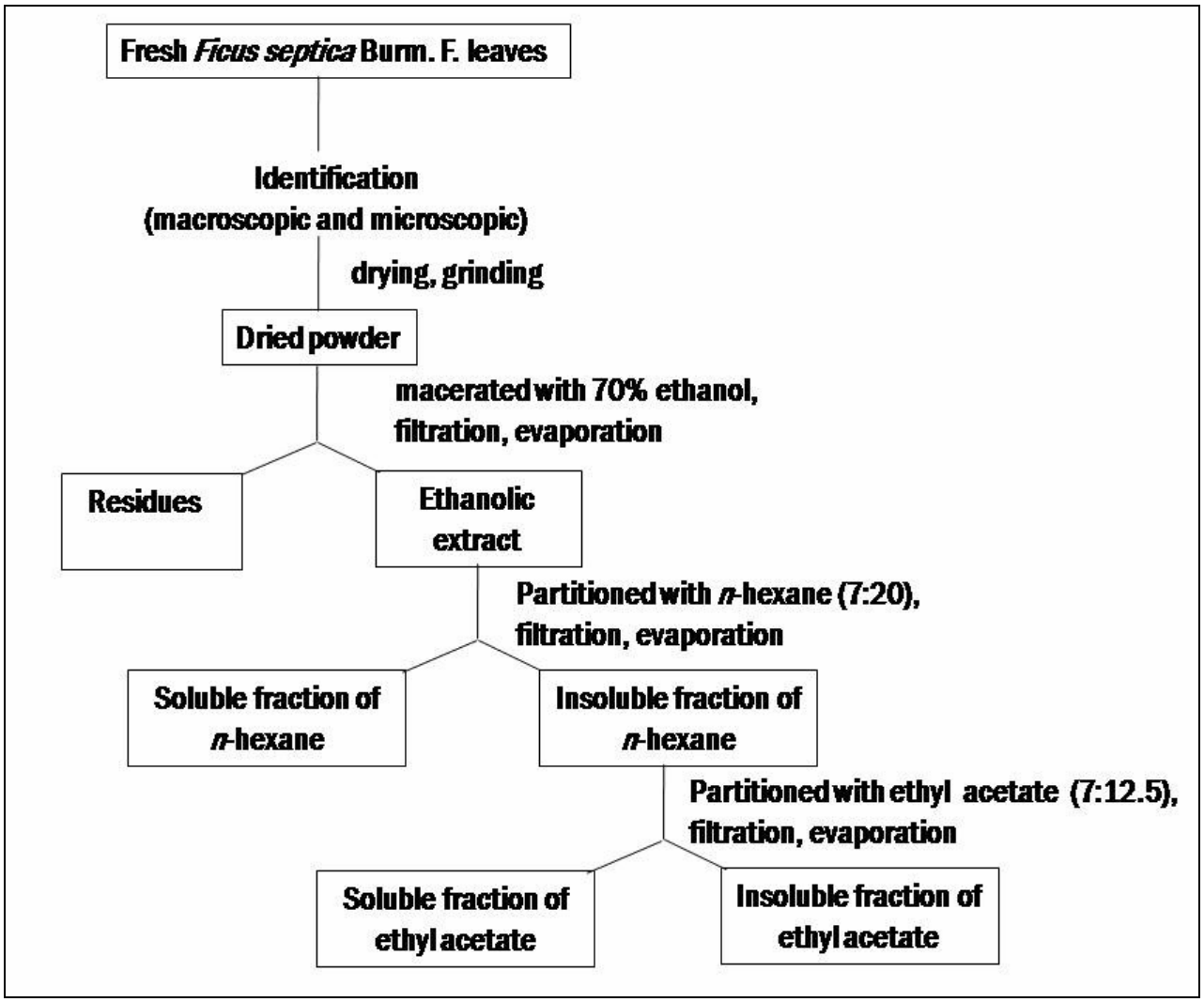

Fig. 1. Scheme of procedures for obtaining fractions of Ficus septica Burm. F ethanolic extract.

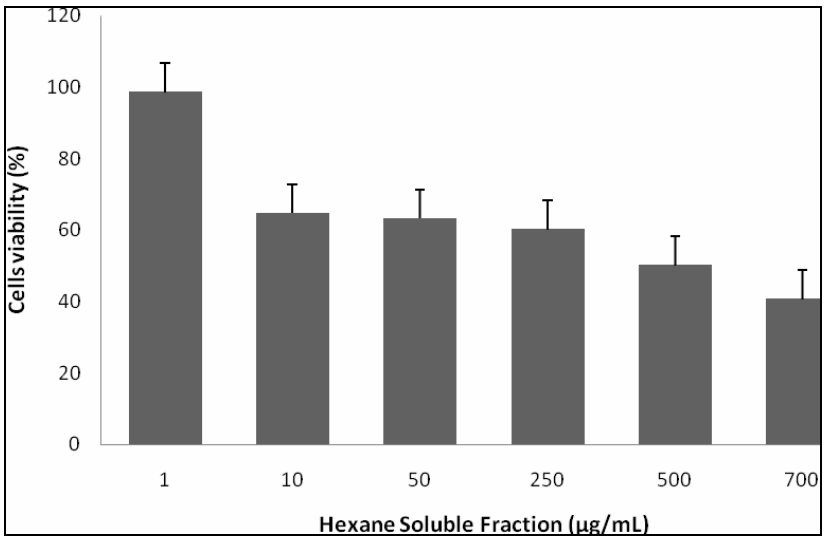

Fig. 2. Effects of hexane soluble fraction of Ficus septica Burm. F ethanolic extract on T47D cell viability. Cells were incubated for 24 hr with various concentrations of hexane soluble fraction. Cell proliferations were examined by MTT assay. Results are the mean ± SEM of three experiments.

Table 1. The IC_{50} values of cytotoxic effect of ethanolic extract fractions of Ficus septica Burm. F ethanolic extract on human breast cancer T47D cell.

Treatment	IC_{50} value (µg/mL)	Inhibitory effect at highest concentration (700 µg/mL)
n-hexane soluble fraction	331.0	59.33±2.41 %
n-hexane insoluble fraction	9.3	88.51±2.22 %
ethyl acetate soluble fraction	13.7	83.78±0.96 %
ethyl acetate insoluble fraction	>700	38.68±1.55%

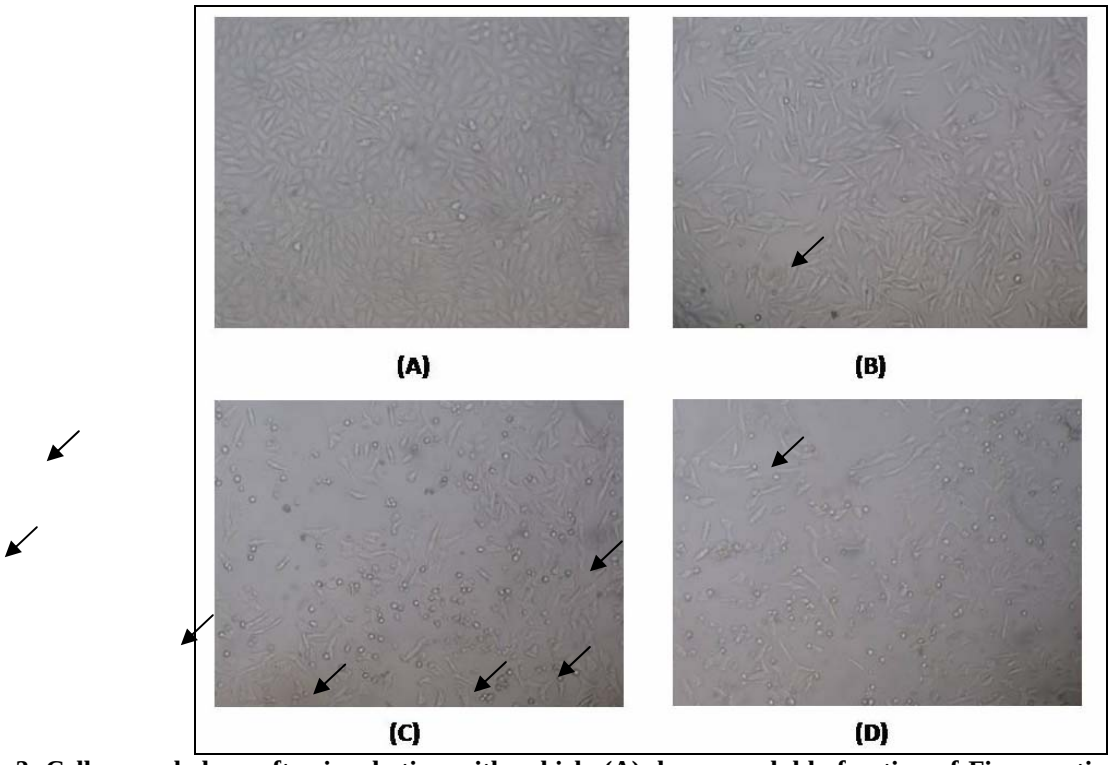

Fig. 3. Cells morphology after incubation with vehicle (A), hexane soluble fraction of Ficus septica Burm. F ethanolic extract at concentration of 50 µg/mL (B), 500 µg/mL (C), and 750 µg/mL (D). Death cell was pointed by black arrow.

Effect of n-hexane insoluble fraction on T47D cell viability

Figures 4-5 show the effects of a series concentration of n-hexane insoluble fraction of Ficus septica Burm. F. leaves on the viability of breast cancer T47D cell lines for 24 hours incubation. The n-hexane insoluble fraction decreased the cells viability potently in concentration-dependent manner. At concentration of 10 µg/mL (low concentration) decreased the the cell viability by 52.33±2.41 %. A decrease of less than 50% at low concentration indicates that the fraction has a potent cytotoxic effect. Whereas at the highest concentration (700 µg/mL), the fraction decreased the cell viability by 88.51±2.22 %. IC_{50} value of n-hexane soluble fraction of Ficus septica Burm. F. leaves was 9.3 µg/mL (Table 1). It indicates that the n-hexane insoluble fraction is more potent than this of soluble fraction.

Therefore, n-hexane insoluble fraction was fractionated by ethyl acetate to yield soluble fraction and insoluble fraction. Subsequently, both fraction were evaluated for their cytotoxic effects. Their effects of a series concentration of these fractions on the viability of breast cancer T47D cell lines for 24 hours incubation were showed in Fig. 6-9.

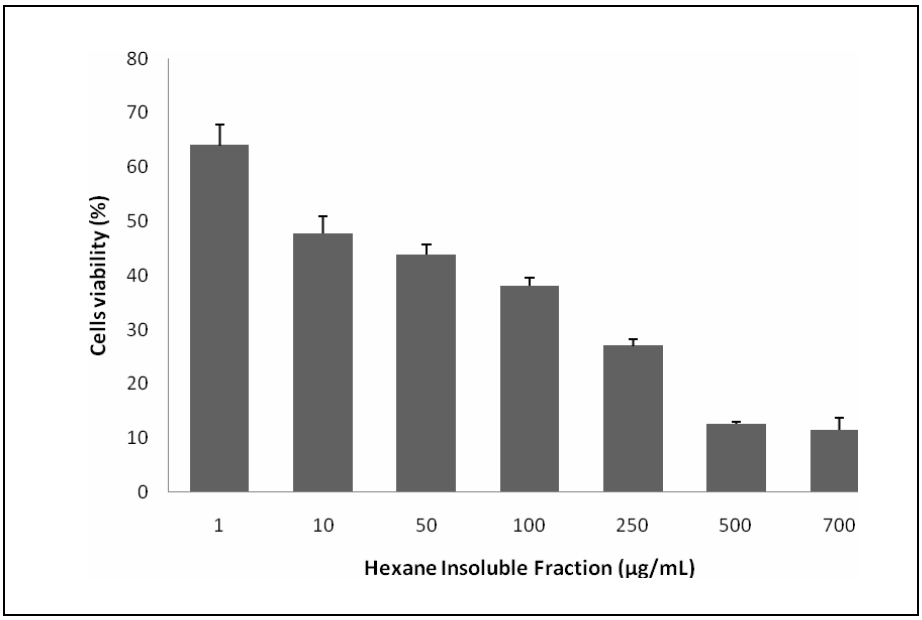

Fig. 4. Effects of hexane insoluble fraction of Ficus septica Burm. F ethanolic extract on T47D cell viability. Cells were incubated for 24 hr with various concentrations of hexane insoluble fraction. Cell proliferations were examined by MTT assay. Results are the mean ± SEM of three experiments.

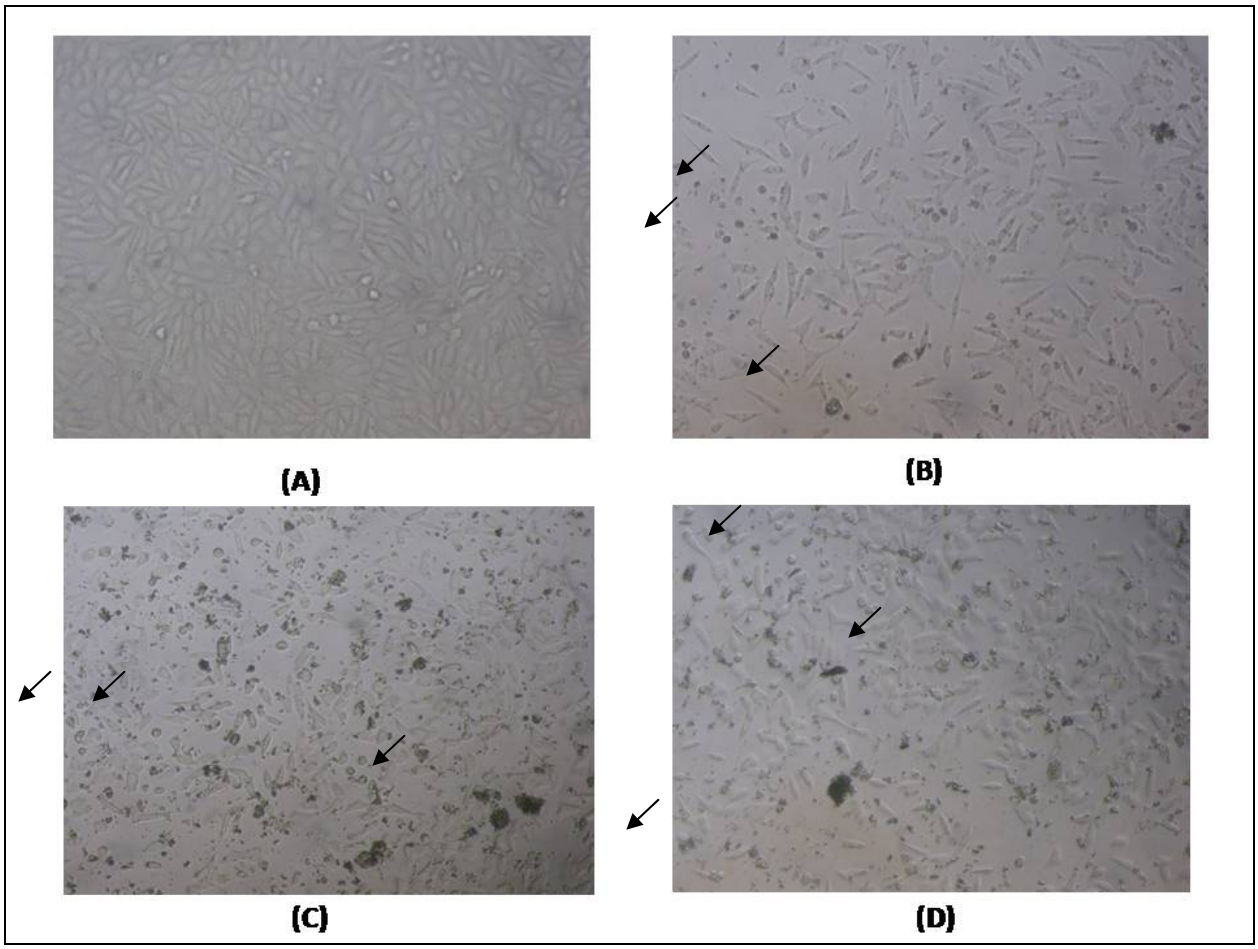

Fig. 5. Cells morphology after incubation with vehicle (A), hexane insoluble fraction of Ficus septica Burm. F ethanolic extract at concentration of 50 µg/mL (B), 500 µg/mL (C), and 750 µg/mL (D). Death cell was pointed by black arrow.

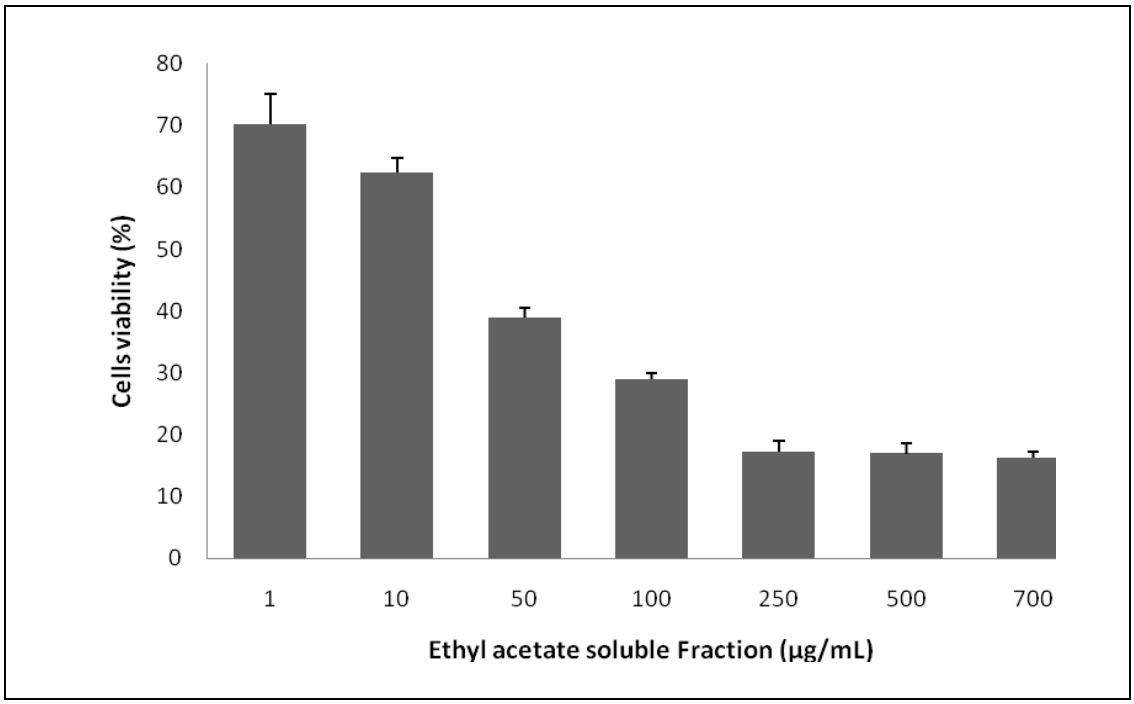

Fig. 6. Effects of ethyl acetate soluble fraction of Ficus septica Burm. F ethanolic extract on T47D cell viability. Cells were incubated for 24 hr with various concentrations of ethyl acetate soluble fraction. Cell proliferations were examined by MTT assay. Results are the mean ± SEM of three experiments.

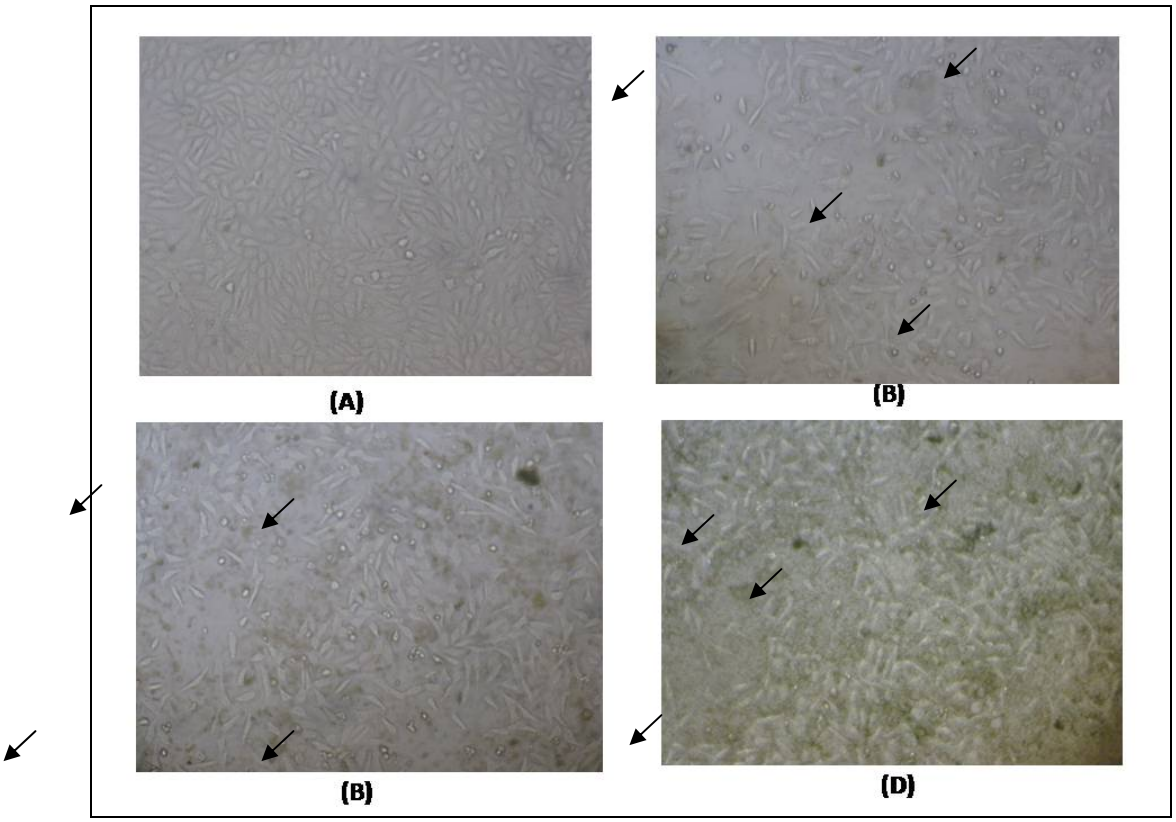

Fig. 7. Cells morphology after incubation with vehicle (A), ethyl acetate soluble fraction of Ficus septica Burm. F ethanolic extract at concentration of 50 µg/mL (B), 100 µg/mL (C), and 250 µg/mL (D). Death cell was pointed by black arrow.

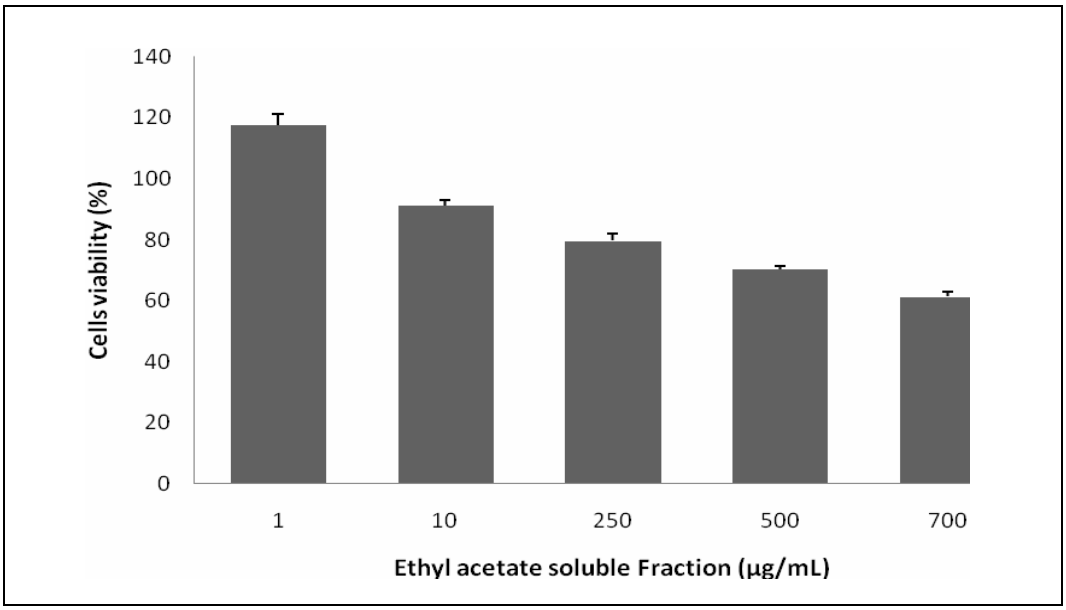

Fig. 8. Effects of ethyl acetate insoluble fraction of Ficus septica Burm. F ethanolic extract on T47D cell viability. Cells were incubated for 24 hr with various concentrations of ethyl acetate insoluble fraction. Cell proliferations were examined by MTT assay. Results are the mean ± SEM of three experiments.

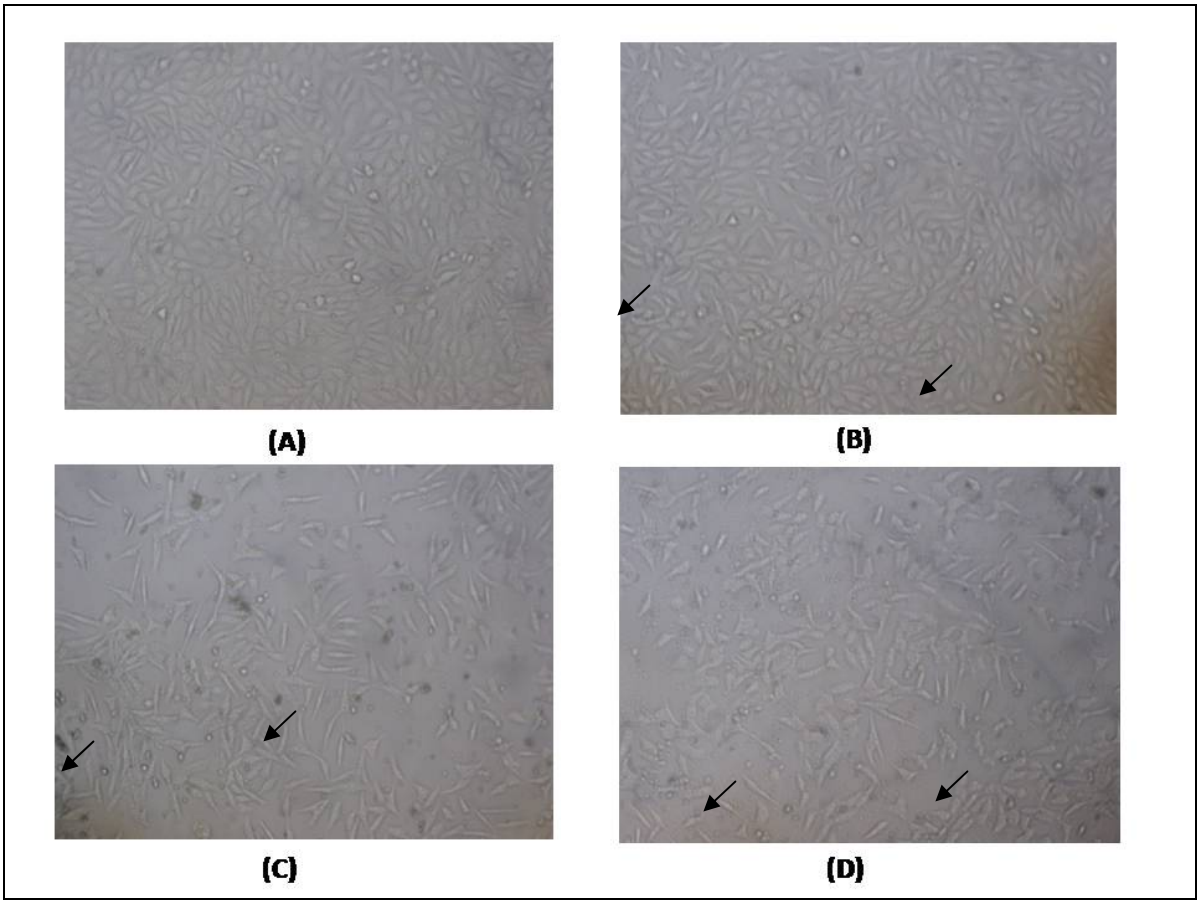

Fig. 9. Cells morphology after incubation with vehicle (A), ethy acetate insoluble fraction of Ficus septica Burm. F ethanolic extract at concentration of 10 µg/mL (B), 250 µg/mL (C), and 700 µg/mL (D). Death cell was pointed by black arrow.

Effect of ethyl acetate soluble fraction on T47D cell viability

Ethyl actate soluble fraction succeeded to decrease the cells viability in concentration-dependent manner. All concentrations used in the experiment could decrease the cells viability significantly ($P<0.05$). At concentration of 50 µg/mL decreased the cells viability up to 60%. A decrease of less than 50% at low concentration indicates that the fraction has a potent cytotoxic effect. At higher concentration (250-700 µg/mL), the fraction strongly depleted the cell viability up to 80% (Fig. 6-7). IC_{50} value of n-hexane soluble fraction of Ficus septica Burm. F. leaves was 13.7 µg/mL (Table 1).

Effect of ethyl acetate insoluble fraction on T47D cell viability

Effect of ethyl acetate insoluble on T47D cell viability was showed in Fig. 8-9. All concentrations used in the experiment could not decrease the cells viability up to 50%. At highest concentration (700 µg/mL), the fraction decreased the cell viability by 38.68±1.55%. IC_{50} value of n-hexane soluble fraction of Ficus septica Burm. F. leaves was more than 700 µg/mL. It indicates that the ethyl acetate insoluble fraction is much less potent than this of the ethyl acetate soluble fraction.

Discussion

Attemps to discover anticancer agent from natural product is increasing along with increasing cases of cancer. Indonesia has the second largest biodiversity in the world including medicinal plants. In Indonesia, exploration of anticancer agents from medicinal plants have been widely studied. Kirana et al. have screened 11 main species of Zingiberaceae from Indonesia for their antitumor effect on human HT-29 colon cancer and MCF-7 breast cancer cells. In this study, Curcuma longa, Kaempferia pandurata and Zingiber aromaticum showed inhibitory activity against both cell lines [9]. Sugiyanto et al. have also screened many Indonesia plants for their anticancer activity. Among the many Indonesian plants studied, sambung nyawa (Gynura procumbens), beluntas (Pluchea indica), murbei (Morus alba) dan tapak doro (Vinca alba) leaves showed anticarcinogenic activity on lung tumor growth of mice [10]. Our research group named Cancer Chemoprevention Research Center (CCRC) has selected several Indonesian plants for their anticancer activity in breast cancer. The plants which is promising to be further investigated were **Brucea janvanica** [11]**, Thyphonium flagelliforme** [12]**, Areca catechu** [13]**,** Vigna sinensis [14], Piper aduncum [15], Cosmos caudatus [16] and Ficus septica [7].

Previous study, ethanolic extract of Ficus septica Burm. F. showed a potent cytotoxic effect on breast cancer T47D cell lines with IC_{50} value of 13 µg/mL [6]. The extract also induced apoptosis in MCF-7 breast cancer cells, and downregulated Bcl-2 protein [7]. In vivo study of the extract on dose of 750 mg/kg BW induced apoptosis throught p53-independent pathway in 7,12-dimethylbenz[a]nthracene-induced rat liver cancer [8].

In present study, we investigated the active fraction of ethanolic extract of Ficus septica Burm. F on breast cancer T47D cell lines. The fractionation was carried out in two stages using n-hexane and ehtyl acetate, respectively, as described in Fig.1. First step fractionation using n-hexane aimed to separate polar and non-polar compounds. The step yielded n-hexane soluble fraction and n-hexane insoluble fraction. The results showed that the IC_{50} value of n-hexane insoluble fraction (9.3 µg/mL) was much less than this of n-hexane soluble fraction (331 µg/mL) (Table 1). It indicates that the cytotoxic effect of n-hexane insoluble fraction was much more potent than this of n-hexane soluble fraction. Based on the results, the active compounds of ethanolic extract of Ficus septica Burm. F might be partitioned in n-hexane insoluble fraction. The next step, hexane insoluble fraction was partitioned with ethyl acetate to separate polar compounds and semi polar yielding ethyl acetate soluble fraction and ethyl acetate insoluble fraction, respectively. The

results showed that the IC_{50} value of ethyl acetate soluble fraction (13.7 µg/mL) was much less than this of ethyl acetate insoluble fraction (>700 µg/mL) (Table 1). It indicates that the cytotoxic effect of ethyl acetate soluble fraction was much more potent than this of ethyl acetate insoluble fraction. From this result, the active compounds of n-hexane insoluble fraction might be partitioned in ethyl acetate soluble fraction, but it need to be explored more details.

The cytotoxic activity of natural products is related to presence of anticancer compound in these plants including Ficus septica Burm. F. Wu et al. found out the active compounds from Ficus septica leaves i.e. phenanthroindolizidine alkaloids. These compounds were ficuseptine, (+)-tylophorine, and a mixture of (+)-tylocrebrine and (+)-isotylocrebrine. These compounds showed potent cytotoxic effect on two human cancer cell lines (NUGC and HONE-1)[17]. These cell lines are gastric adenocarcinoma and nasopharyngeal carcinoma cell lines, respectively. Other types of alkaloids were also found from methanolic extract of Ficus septica leaves and evaluated for cytotoxic effect. The compounds include ficuseptamines A and B (aminocaprophenone alkaloids) and ficuseptamine C (pyrrolidine alkaloid) [18]. The alkaloids were also found in other parts of Ficus septica Burm. F and exhibited potent cytotoxic effect. Phenanthroindolizidine alkaloids could be also isolated from both roots and stems of Ficus septica. These compounds showed cytotoxic effect on NUGC and HONE-1 cell lines [19-20]. Based on the facts, phenanthroindolizidine alkaloids have an important role in the cytotoxic effect of Ficus septica Burm. F. However, the extract compounds on each fraction of Ficus septica leaves ethanolic extract need to be explored further.

Conclusion

Based on the result dan discussion described above, we concluded that n-hexane insoluble fraction and ethyl acetate soluble fraction exhibited potent cytotoxic on breasr cancer T47D cell lines. The fractions are potential to be developed as anticancer agents in breast cancer therapy.

Author's Contribution

AEN was responsible to make a research concept and design of the study, data collection, acquisition of data, analysis of data, statistical of data, drafted and corresponding author the manuscript. MI and AH contributed to providing the ethanolic extract fractions of Indonesia plant Ficus septica Burm. F. DD helped to culture the cells and collect the data. EM participated in drafted the manuscript. All author have already read and approved the final revision of this manuscript.

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