In silico, in vitro and in vivo Tests of Ficus deltoidea Jack Leaves Extract as Inhibitor for β-Catenin Expression in Colon Carcinogenesis Model

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ABSTRACT
Context: Ficus deltoidea Jack leaves extract as anticolorectal cancer. Aims: This study aims to analyze the potential of FD extract to be an anti-colon cancer by investigating the extract capability in reducing β-catenin expression and inhibiting colon cancer cells growth. Settings and Design: The research was conducted in Medical Faculty Universitas Indonesia with experimental design. Methods and Material: FD ethanol extracts was tested in vitro, in silico and in vivo. In vitro test was conducted to human colon cell lines. In vivo test was conducted to Balb/c mice induced with 10 mg/kg azoxymethane (AOM) and dextran sodium sulfate 1% (DSS). The colonic tissue collected was the distal portion. β-catenin expressions in the cytoplasm and nuclei of the epithelial cells of the colon crypt were semi quantitatively assessed using the immunohistochemistry staining on ten visual fields with 400x magnification. Statistical analysis used: SPSS. Results: FD ethanol extracts inhibit the expression of β-catenin in the crypt epithelial cells of mice colon induced with AOM/DSS. The extracts also inhibit the growth of human colon cancer (HCT 116) with IC50 value of 5.41 mg/mL. Phytochemical screening to the extracts gave three groups of compounds: alkaloid, flavonoid, and tannin. Water fraction is the best fraction. Based on in the results of in silico analysis with molecular docking, FD extract is believed to influence the expression of β-catenin, in which vitexin and isovitexin are the main candidate compounds to influence the expression of the protein. Conclusion: FD ethanol extract is potential to be an anti-colon cancer proven by the extract capability to reduce β-catenin expression.
Key words: Azoxymethane, β-catenin, Ficus deltoidea, Colon carcinogenesis, in silico.
Key Message: These article give the new explanation of the effect from Ficus deltoidea extract as anticolorectal agents with in vitro, in silico and in vivo model.

INTRODUCTION
Ficus deltoidea Jack (FD) belongs to the Moraceae family and widely grown in the islands of Java, Sumatra, Kalimantan and Maluku. FD is also found in Malaysia and Thailand, and grow in a small extent in China.1 In Indonesia, FD is known as Tabat Barito for many are found grown well along the Barito river in the South of Kalimantan. In its natural habitat, FD is an Epiphytic grows on another plants or stick to either larger plants or on the rocks upon which it depends for mechanical support but not for nutrients.2,3 In Indonesia, the juice of the leaves is commonly used as a hair tonic, herbal remedy for women after delivery, and herbal medicine for diseases associated with postpartum conditions.4,5 Many researches have been done to this plant related to its anti-inflammatory, anti-diabetic, anti-melanogenic, and anticancer properties.6 An anticancer research that was conducted to ovarian cancer cell lines, in vitro showed IC50 value of 224.39 ± 6.24 pg/mL for FD aqueous extract and 143.03 ± 20.21 pg/mL for FD ethanolic extract.2,8 A more extensive study on FD anticancer potential with other types of cancer, both in vitro and in vivo, needs to be performed. In Indonesia, colon cancer is the third most common cancer diagnosed after cervical and breast cancer. As the etiology of colon cancer is associated with inflammation7 and the sufferer today shifts to younger age, exploration to Indonesian native herbs for the treatment of this cancer is getting more and more attention.9,11 FD contains flavonoid as one of the active compounds.12 Flavonoids are associated with antioxidant properties7 while antioxidants are associated with barrier to genome damage.13,14 One of the genome belongs to tumor suppressor genes is β-catenin, which is an important marker in colon carcinogenesis.15,16 Based on the above, this study was conducted to examine the potential of FD extract on the expression of β-catenin, in silico in vivo and in vitro.
test was conducted to mice, in which the carcinogenesis induced with azoxymethane and dextran sodium sulfate. *In vitro* test was conducted to human colon cell lines.

**MATERIALS AND METHODS**

*Ficus deltoidea* Jack (FD)

Fresh FD plants were obtained from their natural habitat in Tabat Barito Conservation Areas, Mandalawangi Resort, Gunung Gede Pangrango National Park, Bogor. FD grows well in the temperature range of 18.3°C-23.1°C, relative humidity of 80-84%, and in the range offland slope of 4-24% with various slope directions. The plants were identified at the Herbarium Bogoriense, Bogor, Indonesia.

**FD Extract Preparation**

*Ficus deltoidea* fresh leaves (± 1.5 kg) was washed and dried under the sunlight. After the leaves completely dried and free of water, the leaves was grounded to powder using a blender. The powder was then sieved with a 40-mesh sieve and weighed (500 g) and kept in a clean and tightly sealed container.

The leaves powder of *Ficus deltoidea* was put into an erlenmeyer flask, solvent was then added and extraction was performed by sonication for 15 min. The material to solvent ratio was 1:10. To the resulting residue it was added the same solvent and extraction was performed with the same procedure. The macerates were then pooled and dried with a rotary evaporator. The resulting extract was weighed and the yield was determined.

**Flavonoid Content Measurement**

Flavonoid content was measured using the method of Dzolin, *et al.* (2015). Catechin was used as a standard in varying concentrations of 20, 40, 60, 80 and 100 mg/mL. One mL aliquot of the extract and catechin standard solution were each diluted in 4 mL distilled water. To the solutions, it was added with 0.3 mL of 5% NaNO₃ and after 5 min, 0.3 mL 10% AlCl₃ was added. To this mixture, it was added 2 mL NaOH 1M and 2.4 mL distilled water, and mixed thoroughly. The pink color appeared in the solution was measured using UV spectrophotometer at a wavelength of 519 nm. Total flavonoid was calculated based on mg catechin equivalents (mg EqCatechin) per g extract. The measurement was conducted in triplet and the average value was determined.

**Animals**

Experimental animals used were male Balb/c mice, aged 2-3 months, weighing 20-25 g. Mice were bred and reared in the Laboratory of Experimental Pathology, Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia. Mice were kept in a room with temperature of 22±2°C in 12 h light and 12 h dark cycle. Mice were fed with standard pellet and *ad libitum* drinking water. Before being used in the experiment, the mice were confirmed free from parasitic diseases. Animals were reared and treated in accordance with the Guide for the Care and Use of Laboratory Animals of the Animal Care and Use Committee, and had gained approval from the Research Ethics Committee of the Faculty of Medicine, University of Indonesia. Animal rearing and treatment were carried out by Felasa certified researchers.

**Induction of Colon Carcinogenesis**

Induction of colon carcinogenesis in mice was performed according to the method developed by Tanaka, *et al.* (2003) and Suzuki, *et al.* (2006). Mice were injected intraperitoneally with azoxymethane (AOM/Sigma) dissolved in 0.9% NaCl at a dose of 10 mg/kg of body weight for one administration. Post-AOM induction the mice were given standard feed and mineral water for one week. For the next one week, the water was replaced with aquaest containing 1% dextran sodium sulfate (DSS/ Sigma). The mice were reared for first, second, third, and forth month until the time of sacrifice to get the colon tissue. The colonic tissue collected is the distal portion.

**FD Extract Administration**

FD extract was administered orally to each mouse from the third week or after the completion of DSS administration. Mice were divided into 4 groups, each group consisting of 6 animals. The mice grouping are as follows: the first group or negative control group is the group of mice induced with AOM/DSS and only received distilled water during the treatment. The second group is mice induced with AOM/DSS and received 12.5% w/v (low dose) of FD *ad libitum*. Group 3 received 25% w/v (medium dose) of FD, while group 4 received 50% w/v (high dose) of FD. At the end of first, second, third, and forth month after FD administration, the mice were sacrificed.

**Immunohistochemistry staining of β-catenin Staining**

Mice were sacrificed using ketamine-xylazine 16 weeks post-carcinogenesis induction with AOM. Mice colon were removed, cleaned with water, and fixed with 10% phosphate buffered formalin. Distal colon tissue sections were embedded in paraffin. Tissues were cut to a thickness of 4 μm for immunohistochemistry staining. After deparaffination and rehydration, the slides were immersed into 0.01 M citrate buffer (pH 6.0) in microwave for 5 min. 3% H₂O₂ were added in drops for 5 min at room temperature to remove endogenous peroxide. The slides were then incubated with rabbit polyclonal β-catenin antibody (dilution 1:250; Abcam, Inc., Cambridge, MA) in PBS for 2 h at room temperature in humidity chamber, followed by incubation overnight at 4°C. N-Universal (Dako) was used as negative control. The slides were then incubated with an appropriate secondary antibody for 1 h at room temperature followed by incubation with HRP-conjugated streptavidin for 30 min. Protein was visualized using 3, 3′-diaminobenzidine (DAB) for 10 min at room temperature. Harris hematoxylin, a counterstain, was then added, dehydrated and mounted.

**Immunohistochemistry Staining Interpretation**

β-catenin expressions in the cytoplasm and nuclei of the epithelial cells of the colon crypt were semi quantitatively assessed using the criteria of Walker on ten visual fields with 400x magnification. Expressions were reported according to the following score: 0 = negative, 1 = <10% of cells stained, 2 = 10-50% of cells stained, 3 = 50-80% of cells stained, 4 = 80-100% of cells stained. The resulting scores were summed up and the average value was determined (Walker, 2006). Human colonic adenocarcinoma tissue was used as positive control forβ-catenin staining and the tissue samples that were not treated with the primary antibody were used as negative control.

**In vitro experiments**

In this study, *in vitro* experiment used human colon cancer cell line HCT 116 obtained from the Research Institute for Fisheries and Maritime Affairs, Republic of Indonesia. Cells were maintained in CO₂ incubator with 5% CO₂ level, temperature of 37°C in RPMI medium, 5% FCS, 1% penicillin and streptomycin. Confluent cells were harvested by the addition of 0.5% trypsin. Only confluent cells used in the toxicity test using MTT method. This method checks for mitochondrial dehydrogenase enzyme in living cells. The enzyme will reduce the yellow MTT reactant to blue formazan. First, the extract samples were diluted and added to the wells of a 96-well plate. The extract samples were diluted to concentrations of 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml and 800 μg/ml, and each
was made in triplet. After 48 h, the supernatant was removed and the precipitate was washed using PBS (phosphate buffered saline) and then incubated with 10 µM MTT reactant (5 mg/mL) in RPMI 1640 medium for 4 h at 37°C. The reaction was then centrifuged at 500 rpm/min for 5 min. The supernatant was removed again and 100 µL ethanol was added to dissolve formazan crystals. Afterwards, the absorbance (OD) was read at 595 nm and the absorbance of the cells in the control group was considered as 100%. OD values are directly proportional to the number of cells, which means the smaller the OD value, the smaller the number of the living cells.

The resulting OD values showed the number of the living cells. Percent inhibition of each concentration of the treatment is calculated by using the following formula:

\[
\% \text{ inhibition} = \frac{\text{OD control} - \text{OD treatment}}{\text{OD control}} \times 100
\]

Percent inhibition is calculated for each well, and the mean value for every triplet is determined. IC_{50} is obtained from the linear equation of the %inhibition against concentration graph. The intercept and the capability to inhibit 50% cell growth were observed as a measure for cytotoxicity.\(^2\)

### RESULTS

#### In silico Test

Two-dimensional modelling of compounds in FD leaves extracts was conducted by using the database in www.chempider.org. The search for compounds in the PubMed database resulting in, among others, vitexin, isovitexin, epi (catechin), and epi (afzelechin). The results for molecular fixation of protein ligands/targets (the compounds contained in the FD extract) can be seen in Table 1.

#### Phytochemical Test

Phytochemical screening to the ethanol extract of FD was conducted by using the database in www.chempider.org. The search for compounds in the PubMed database resulting in, among others, vitexin, isovitexin, epi (catechin), and epi (afzelechin). The results for molecular fixation of protein ligands/targets (the compounds contained in the FD extract) can be seen in Table 1.

### DISCUSSION

#### Phytochemical Test

The results for phytochemical screening tests of three FD extract fractions can be seen in Table 2. From the n-hexane fraction, it was only obtained triterpenoid compounds, while ethylacetate fraction only contained tannins. However, the water fraction contained alkaloids. Flavonoids and tannins. Several phytochemical studies reported that *Ficus deltoidea*

<table>
<thead>
<tr>
<th>Target</th>
<th>Substances</th>
<th>∆G (Kcal/mol)</th>
<th>pKi (µM)</th>
<th>H donor</th>
<th>H acceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK3β</td>
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<tr>
<td>APC</td>
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<td>11.33</td>
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<td>4</td>
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<tr>
<td></td>
<td>catechin</td>
<td>-10.85</td>
<td>10.07</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>afzelechin</td>
<td>-10.31</td>
<td>7.92</td>
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<td>3</td>
</tr>
<tr>
<td>Axin</td>
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<td>-14.00</td>
<td>9.30</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>isovitexin</td>
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<td>11.39</td>
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<td>3</td>
</tr>
<tr>
<td>Wat</td>
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<td>9.69</td>
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<td>2</td>
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<td>1</td>
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<tr>
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<td>10.58</td>
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<td>MAPK</td>
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<td>8.23</td>
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</table>
contains tannins, flavonoids, alkaloids, and carbohydrates. Tannins may be extracted from all parts of the plant, including leaves, branches, stems, roots and fruits. FD is one of the polyphenol-rich plants, which shows in the high content of total phenol and flavonoid.

**In silico and in vitro Tests**

Table 1 listesome most potential candidate-compounds contained in the FD extract that have strong interaction with the target protein involved in β-catenin pathway in the pathogenesis of colon cancer. The interaction stability can be seen from the Gibbs free energy (ΔG), pKi, and the number of H-donors and H-acceptors. Gibbs free energy (ΔG) represents the strength of the binding affinity of the ligand to the protein. The interaction between ligand and protein can occur if the resulting ligand-protein complex has ΔG value lower than zero. The smaller the ΔG value, the more stable the complex. Stable interaction between the ligand and the target protein is expected to affect the protein performance that will ultimately produce a pharmacological effect.
noticeably high cytotoxicity is associated with phytochemical test results, which showed that FD contains tannins, flavonoids and alkaloids.

**β-catenin expression in in vivo test**

Positive β-catenin expression is indicated by brown staining of the sample preparation. Scoring to the crypt epithelial cells positive to beta-catenin gave an average score value from ten visual fields of 2 (moderate).

As shown in Figure 3, the expression occurs in the cytoplasm and the cell membrane. From Table 4, it is shown that colon carcinogenesis occurred in induced mice of the negative control group. In the low-dose group, colon carcinogenesis is not much affected regardless of the duration of FD extract administration. However, in the medium and high dose groups, there is positive progress over time of administration. This is also the case with the positive control group. FD extract administration shows positive progress starting from the second month (p = 0.024) continues to the third month (p = 0.046) and the fourth month (p = 0.022).

**CONCLUSION**

FD ethanol extracts inhibit the expression of β-catenin in the crypt epithelial cells of mice colon induced with AOM/DSS. The extracts also inhibit the growth of human colon cancer (HCT 116) with IC\textsubscript{50} value of 5.41 mg/mL. Phytochemical screening to the extracts gave three groups of compound: alkaloid, flavonoid, and tannin. Water fraction is the best fraction. Based on in silico analysis with molecular docking, FD extract is believed to influence the expression of β-catenin, in which vitexin and isovitexin are the main candidate compounds to influence the expression of the protein.

**CONFLICTING INTEREST**

The authors declare no conflict of interest.

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**ABBREVIATIONS**

FD- Ficus deltoidea; AOM- Azoxymethane; DSS- Dextran Sodium Sulfate; MTT-3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; OD- Optical Density.
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