

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

Kusmardi Kusmardi¹, Tedjo Aryo², Wuyung Puspita Eka¹, Fadilah Fadilah², Priosoeryanto Bambang Pontjo³, Fachri Wilzar^{4*}

Kusmardi Kusmardi¹,
Tedjo Aryo², Wuyung
Puspita Eka¹, Fadilah²,
Priosoeryanto Bambang
Pontjo³, Fachri Wilzar^{4*}

¹Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.

²Department of Chemistry, Faculty of Medicine Universitas Indonesia, Jakarta, INDONESIA.

³Laboratory of Pathology, Faculty of Veterinary, Institut Pertanian Bogor, Bogor, INDONESIA.

⁴Drug Development Research Center, Indonesian Medical Education and Research Institute, Faculty of Medicine Universitas Indonesia, INDONESIA.

Correspondence

Wilzar Fachri

Salemba Raya Street, 6th, IMERI Building Medical Faculty 11th Floor, Universitas Indonesia, INDONESIA.

Phone no: +62 21 31930302

E-mail: wilzar.fachri01@webmail.ui.ac.id

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ABSTRACT

Context: *Ficus deltoidea* Jack leaves extract as anticancer. **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 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 anticancer 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.^{6,7} An anticancer research that was conducted to ovarian cancer cell lines, *in vitro* showed IC₅₀ values 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 inflammation⁹ and the sufferer today shifts to younger age, exploration to Indonesian native herbals for the treatment of this cancer is getting more and more attention.^{10,11}

FD contains flavonoid as one of the active compounds.¹² Flavonoids are associated with antioxidant properties,² 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*. *In vivo*

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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 of land slope of 4-24% with various slope directions.¹⁷ The plants were identified at the Herbarium Bogoriense, Bogor, Indonesia.

FD Extract Preparation

Ficus deltoidea fresh leaves (\pm 1.5 kg) was washed and dried under the sunlight. After the leaves completely dried and free of water, the leaves were 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.¹⁸

Flavonoid Content Measurement

Flavonoid content was measured using the method of Dzolin, et al. (2015).¹² 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% NaNO₂ 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.¹²

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 \pm 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.¹⁹ 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 aquadest containing 1% dextran sodium sulfate (DSS/Sigma). The mice were reared for first, second, third, and fourth month until the time of sacrifice to get the colon tissue. The colonic tissue collected is the distal portion.²⁰

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 fourth 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 μ m for immunohistochemistry staining. After deparaffinization 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 for β -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.²¹ 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 μ g/ml, 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, 100 μ g/ml, 200 μ g/ml, 400 μ g/ml and 800 μ g/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 μ l 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 μ l 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. IC₅₀ 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.²²

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 three fractions: hexane, ethyl acetate, and water. Water fraction resulting in three groups of compounds: alkaloids, flavonoids, and tannins. Water fraction is the best of the three (Table 2). Total flavonoid content is reported in mg equivalent of catechin (mg Eq Catechin) per g of extract and the results from three measurements are 278, 302 and 314 mg Eq Catechin/gram with an average of 298 mg Eq Catechin/gram.

In vitro Test

Table 3 shows the results of optical density for each group of dose performed in triplet. From the results of optical density, it was then determined the percent inhibition for each group. The group for dose treatment of 6.25 μ g/ml had percent inhibition of 82.19%, 12.5 μ g/ml (93.77%), 25 μ g/ml (94.77%), 50 μ g/ml (94.40%), 100 μ g/ml (93.77%), 200 μ g/ml (92.53%), 400 μ g/ml (92.90%), and 800 μ g/ml (90.66%).

Figure 1 presents microscopic observation under Dino Eye microscope for HCT 116 cells, which were treated with various concentrations of FD leaves extract.

IC₅₀ is calculated using Microsoft Excel 2013 by connecting the FD extract concentration (x-axis) and the percent inhibition (y-axis), from which a linear equation was then created (Figure 2).

Based on the line equation in Figure 2, IC₅₀ can be calculated as follows:

$$\begin{aligned} 50 &= 3.2006x + 32.676 \\ 50 - 32.676 &= 3.2006x \\ 17.324 &= 3.2006x \\ x &= \frac{17.324}{3.2006} = 5.412735 \sim 5.41 \mu\text{g/ml} \end{aligned}$$

Thus, it is obtained IC₅₀ = 5.41 μ g/ml

The Expression of β -catenin

Generally, β -catenin expressions in the colon tissue of mice after FD extract administration were found in the cytoplasm and the cell membrane (Figure 3). Based on the number of brown-stained crypt epithelial cells of mice colon, the expressions were scored 0-4. Score 0 = negative, 1 = <10% cells stained, 2 = 10-50% cells stained, 3 = 50-80% cells stained, and 4 = 80-100% cells stained.

Table 4 shows the average score for β -catenin expression in the crypt epithelial cells of mice colon after FD extract administration with various doses and in various duration of administration.

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 1: Stability of Gibbs free energy interaction (ΔG), inhibition constants (pKi), and the number of H-donor and H-acceptor between the target protein and a candidate compound of FD extract.

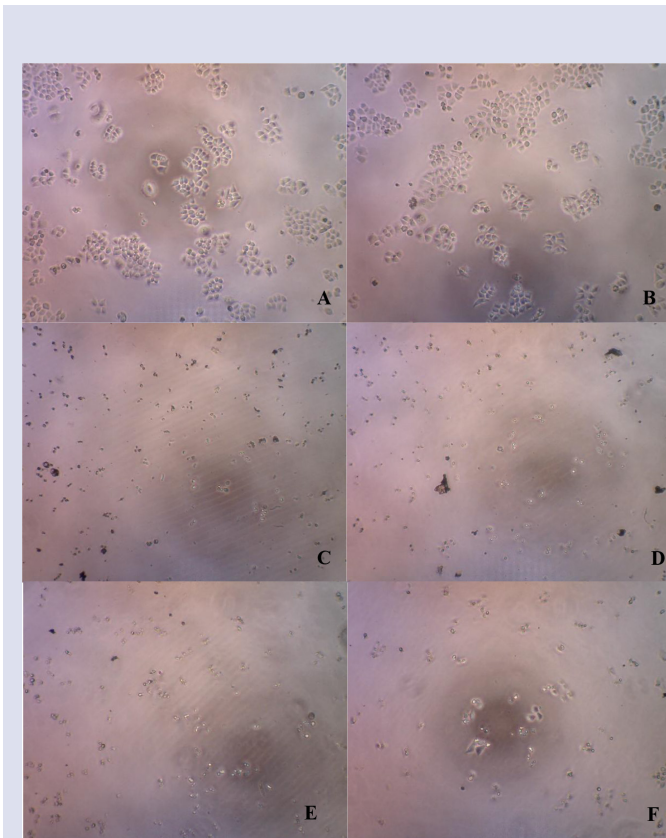
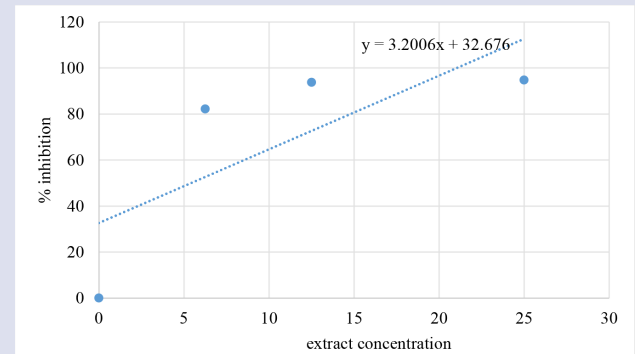
Target	Substances	ΔG (Kcal/mol)	pKi (μ M)	H donor	H acceptor
GSK3 β	vitexin	-13.99	9.98	1	4
	isovitexin	-11.47	7.71	1	0
	catechin	-12.29	8.61	1	1
APC	vitexin	-11.72	11.33	4	4
	catechin	-10.85	10.07	4	3
	afzelechin	-10.31	7.92	0	3
Axin	vitexin	-14.00	9.30	3	3
	isovitexin	-14.60	11.39	5	3
Wnt	vitexin	-1.28	9.69	3	2
	isovitexin	-10.97	5.70	1	1
B-catenin	vitexin	-11.14	10.58	5	6
NFkB	afzelechin	-11.48	5.46	0	1
MAPK	vitexin	-11.24	7.70	5	2
	isovitexin	-9.79	4.40	1	2
PPAR α	vitexin	-11.90	8.23	2	4

Table 2: Results of Phytochemical Screening of *Ficus deltoidea* Fractions.

Substances	Hexane Fraction	Ethyl Acetate Fraction	Water Fraction	Color
Alkaloid	-	-	+	Broken white
Flavonoid	-	-	+	Reddish-yellow
Tanin	-	+	+	Blue-black
Triterpenoid	+	-	-	Red or purple
Saponin	-	-	-	Foam formed for 10 min

Table 3: Percent Inhibition of HCT 116 after incubation for 24 h.

FD Extract	OD I	OD II	OD III	Median	Min.	Max.	Inhibition (%)
Control	0.269	0.281	0.289	0.281	0.269	0.289	0.00
6.25	0.033	0.057	0.053	0.053	0.033	0.057	82.19
12.5	0.019	0.012	0.019	0.019	0.012	0.019	93.77
25	0.014	0.015	0.013	0.014	0.013	0.015	94.77
50	0.016	0.014	0.015	0.015	0.014	0.016	94.40
100	0.020	0.014	0.016	0.016	0.014	0.02	93.77
200	0.021	0.019	0.02	0.02	0.019	0.021	92.53
400	0.019	0.012	0.019	0.019	0.012	0.019	92.90
800	0.028	0.023	0.024	0.024	0.023	0.028	90.66

**Figure 1:** HCT116 cells observation using Dyno Eye microscope. A. Control 1, B. Control 2, C. FD Extract Administration of 50 ppm, D. 25 ppm, E. 12.5 ppm, and F. 6.25 ppm.**Figure 2:** Linear Equation for the determination of IC_{50} of FD leaves ethanol extract On HCT 116 Cells.

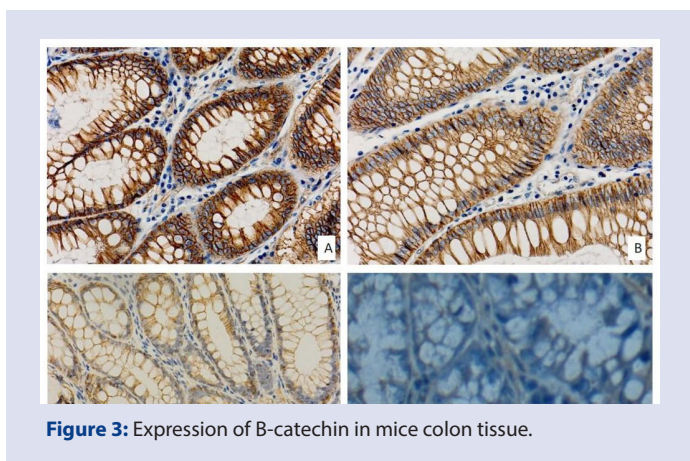
contains tannins, flavonoids, alkaloids, and carbohydrates. Tannins may be extracted from all parts of the plant, including leaves, branches, stems, roots and fruits. FD is one of the polyphenol-rich plants, which shows in the high content of total phenol and flavonoid.

In silico and *in vitro* Tests

Table 1 listed some 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.

Table 4: Average score for β -catenin expression after FD extract administration.

Group	Duration of Extract Administration (month)				P
	1	2	3	4	
Negative Control	1.42	1.64	2.46	3.35	0.068
Low dose	1.46	1.26	1.98	2.68	0.242
Medium dose	1.06	1.22	1.10	2.14	0.036
High dose	0.68	1.04	0.42	0.44	0.026
Positive Control	0.98	1.24	1.26	0.22	0.019
P	0.246	0.024	0.046	0.022	

**Figure 3:** Expression of B-catechin in mice colon tissue.

The other parameter, inhibition constant (pK_i), can give an overview on the affinity between the ligand and the target protein. The smaller the K_i value the better the result, which means the smaller the K_i value, the equilibrium of the reaction tends to shift toward the complex formation. Binding energy (ΔG) value and K_i has a proportional relationship and the value follows the thermodynamic equation below:

$$\Delta G = -RT \ln K_i$$

A protein-ligand complex is said to have good binding affinity if the K_i value is in micro molar range. In addition to the value of Gibbs free energy (ΔG), the interaction stability also influenced by the number of the hydrogen bond formed. Based on the rules of Lipinski, good permeability and absorptivity of a compound will be achieved if the interaction between a compound and a protein receptor is formed by less than 5 (five) H-donor and H-proton.

These hydrogen bonds contribute to the affinity of the ligand to the enzyme/protein due to electrostatic interaction between oxygen or nitrogen atom of the ligand and the hydrogen atom of the amino acid residue of the enzyme/protein or vice versa. The interaction between the protein and the ligand in the active site of the protein is also influenced by these electrostatic and steric factors. This interaction will determine the ΔG value, so that it is possible to estimate the most stable protein-ligand complex.

From Table 1, based on the results of prediction by molecular docking, the extract of *Ficus deltoidea* is believed to affect the expression of β -catenin, in which vitexin and isovitexin are the main candidate compounds influencing the expression of the protein.

From the *in vitro* experiment against the human colon cancer cells (HCT 116), it is seen that FD has a relatively high cytotoxic activity. This

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_{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 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.

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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ABOUT AUTHORS



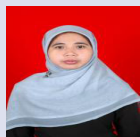
Kusmardi (M.Sc. PhD): Lecturer and Researcher at Pathological Anatomy and Drug Development Research Center Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia. Research interest in Immunology, Pathology, Cancer Biology, Herbal Medicine, Biomedical Sciences, Oncology, Animal Model for Cancer Research, Biostatistic, and Research Methodology.



Aryo Tedjo (S.Sc., M.Sc.) :Lecturer and Researcher at Medical Chemistry and Drug Development Research Center Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia. Research interest in Analytical Chemistry and Herbal Medicine.



Puspita Eka Wuyung (M.Sc., PhD) Lecturer and Researcher at Pathological Anatomy, Faculty of Medicine, Universitas Indonesia. Research interest in Cancer Biology, Herbal Medicine, Biomedical Sciences, Oncology and Animal Model for Cancer Research.



Fadilah (M.Sc., PhD) :Lecturer and Researcher at Medical Chemistry and Drug Development Research Center Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia. Research interest in Bioinformatics, Analytical Chemistry and Herbal Medicine.



Prof. dr. Bambang Pontjo Priosoeryanto (MS., PhD., APVet): Lecturer and Researcher at Animal Health and Veterinary Education Policy, Faculty of Veterinary Medicine, Bogor Agricultural University. Research interest Veterinary Pathology with specialization of Tumor Pathology, Tissue Culture, Diagnostic Pathology.



Wilzar Fachri, (M.Sc.) Researcher at Drug Development Research Center Cluster, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia. Research interest in tissue culture and animal models in drug development.

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