

Antiproliferative activity and caspase enhancement properties of *Annona muricata* leaves extract against colorectal cancer cells

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ABSTRAK

Latar belakang: Prevalensi kanker kolorektal meningkat di Asia termasuk di Indonesia. *Annona muricata* secara tradisional digunakan untuk menjaga kesehatan dan akhir-akhir ini digunakan untuk pasien kanker. Tujuan penelitian ini untuk mengetahui efek antiproliferatif ekstrak daun *A. muricata* dan kemampuannya meningkatkan aktivitas caspase *in vitro* dan *ex vivo*.

Metode: Tiga puluh pasien kanker kolorektal secara acak dikelompokkan untuk menerima 300 mg ekstrak daun *A. muricata* (n=15) atau plasebo (n=15), setiap hari selama 8 minggu. Setelah itu serum pasien kanker kolorektal dari kedua kelompok digunakan untuk penelitian *ex vivo* untuk mengetahui efek ekstrak terhadap aktivitas caspase-8 dan caspase-9 cell line kanker kolorektal. Efek antiproliferatif ekstrak air daun *A. muricata* dan fraksinya dievaluasi terhadap cell line kanker kolorektal (DLD-1 dan COLO 205) dengan alat tes mikrokultur berdasarkan pengurangan metabolisme 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dibandingkan dengan 5-fluorourasil dengan dosis 62,5-2.000 µg/mL atau dengan plasebo. Data diuji dengan uji U Mann-Whitney. Batas kemaknaan ditetapkan pada nilai p≤0,05.

Hasil: Fraksi larut etanol dari ekstrak air daun *A. muricata* menunjukkan efek sitotoksik pada sel kanker kolorektal DLD-1 dan COLO 205, dengan konsentrasi hambatan lebih rendah dibanding plasebo dan 5-fluorourasil, masing-masing 20,59 µg/mL dan 654,9 µg/mL. Serum pasien yang minum ekstrak, bermakna dalam menstimulasi aktivitas caspase 9 (p=0,001) pada DLD-1 cell line kanker kolorektal, namun tidak bermakna dalam menstimulasi aktivitas caspase 8 (p=0,372).

Kesimpulan: Hasil penelitian menunjukkan potensi sitotoksitas ekstrak daun *A. muricata* dalam penelitian *in vitro* dan *ex vivo* kanker kolorektal.

ABSTRACT

Background: The prevalence of colorectal cancer is rising in Asia including Indonesia. *Annona muricata* tea leaves, that is traditionally used for maintaining health, and lately being used by cancer patients. The objectives of this study is to investigate its effects in human colorectal cancer cell *in vitro* and *ex vivo*.

Methods: Thirty patients with colorectal cancer (CRC) were enrolled in a randomized double-blind placebo-controlled trial. They were equally divided into two groups: those treated with 300 mg *A. muricata* leaf extract and placebo daily for 8 weeks. Serum from supplemented CRC patients of both groups was compared for caspase 9 and caspase 8 enhancement activity. Antiproliferative effect of water extract of *A. muricata* leaves and its fractions were evaluated against colorectal cancer cell line (DLD-1 and COLO 205) compared with 5-fluorouracil and placebo, the dose range was 62.5-2,000 µg/mL. Method used was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data were analyzed by Mann-Whitney U test. The p value was set at 0.05.

Results: Ethanol-soluble fraction of *A. muricata* leaves extract water extract (ESFAM) leaves extract had cytotoxicity effects on DLD-1 as well as COLO 205 cell line, as shown by the lower IC₅₀ compared to 5-fluorouracil and placebo, 20.59 µg/mL and 654.9 µg/mL, respectively. Serum of subjects supplemented with extract significantly induced caspase 9 (p=0.001) activity of DLD-1 colorectal cancer cell line, but not for caspase 8 activity (p=0.372).

Conclusion: The study's results suggest the cytotoxicity potential of *A. muricata* leaves extract in *in vitro* and *ex vivo* studies.

Keywords: *A. muricata* leaves, cytotoxicity, colorectal cancer cell line, *ex vivo*, *in vitro*

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The prevalence of colorectal cancer is rising in Asia¹ and it is now the third most common malignant disease in both men and women. There are two to four folds increase of cancer incidence in the last five years.² In Indonesia colorectal cancer was recently estimated to be the five most prevalent cancer found in males and females among 13 cancer registries.³

Many types of cancer have the mechanism to avoid apoptosis induced by anticancer drugs.⁴ The intervention of multistage carcinogenesis by regulating intracellular signaling pathways may give molecular basis of chemoprevention with a wide variety of dietary phytochemicals.⁵ Therefore, there is a growing interest to explore the possibility of using phytochemicals such as the ones found in our diet as chemopreventive agents.

Members of family *Annonaceae* have been investigated as potential sources of biologically active Annonaceous acetogenins, some of which has demonstrated a powerful anti-tumor activities.⁶ Currently, 34 acetogenins have been identified in the leaves of *Annona muricata* Linn.⁷ People have used *A. muricata* leaves traditionally by brewing in hot water and this preparation is found to be safe.⁸ The cytotoxicity of acetogenins has been shown to be stronger in tumorous than in normal cells.⁹ The primary site of action of the acetogenins is complex I of the electron transport chain in mitochondria.¹⁰ Study in mice showed that annonacin inhibited the normal growth of lung tumors during two-weeks period.¹¹

Phytochemical assessment of *A. muricata* leaves showed the presence of alkaloids, tannins, flavonoids, saponins, anthraquinones and cardiac glycosides, ellagic acid, polyphenolic compounds, triterpenoids, β -sistosterol.¹²⁻¹⁴ Polyphenols such as flavonoid quercetin and flavone (2-phenyl-4H-1-benzopyran-4-one) may have chemoprevention property by reducing the incidence of many types of cancers, especially colon cancer.⁴

Caspase-3 activity and deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assessment showed that the ethanolic extract of *A. muricata* induced apoptosis in the myelogenous leukemic K562 cell line. *A. muricata* is considered to be a potential candidate for the development of pro-apoptotic drugs.¹⁴ Based on

the findings, it is interesting to study the effect of *A. muricata* leaves extract towards cell proliferation as well as towards caspase-8 and caspase-9 in colorectal cancer cell *in vitro*. This study was the first *ex vivo* experiment using serum of colorectal cancer (CRC) patients after eight weeks treatment with the plant extract.

METHODS

The subjects for the *ex vivo* trial were CRC patients receiving outpatient care at the Cipto Mangunkusumo Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia, after primary tumor resection. The protocol of this study was approved by Medical Ethics Committee, Faculty of Medicine, Universitas Indonesia (No. 406/H2.F1/ETIK/2013). Participation in the study was voluntary, and written informed consent was obtained prior to the study. The clinical trial is registered on ClinicalTrials.gov under the identifier NCT02439580.

Male and female CRC patients older than 30 years who had undergone primary tumor resection and were willing to take one capsule per day of *A. muricata* extract or a placebo as an additional treatment throughout the study period were included in the study. In addition, the patients were required to have satisfactory hematological and biochemical function and Karnofsky performance status of $\geq 60\%$.

Patients with the following conditions were excluded from the study: uncontrolled hypertension (untreated systolic blood pressure >160 mm Hg, or diastolic blood pressure >95 mm Hg); serious heart problems; upper limit of serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), and creatinine were 111 U/L, 123 U/L, 3.6 mg/dL respectively; a disability rendering them unable to communicate verbally; or a history of cancers other than colorectal (such as non-melanoma skin cancer, basal cell carcinoma, and squamous cell carcinoma) in the past five years. Pregnant or lactating women, and those not using adequate contraception, were also excluded. In addition, patients taking other investigational drugs, patients with hereditary non-polyposis colorectal cancer (HNPCC), and patients taking probiotic supplementation during the study period were

also excluded to avoid potentially conflicting conditions and treatments.

***Annona muricata* L. extract**

The *A. muricata* extract used in this study is a standardized vacuum dried extract (Zirzak Orac) produced by Javaplant, Central Java, Indonesia, containing 0.018% acetogenin (w/w). Zirzak Orac underwent further fractionation using ethanol to produce ethanol-soluble fraction of *A. muricata* leaves water extract (ESFAM). ESFAM contains 0.36% acetogenin (w/w) or 3.6 mg/g, and a 10 g water extract is equivalent to a 2 g ethanolic fraction.

In this study, the CRC patients consumed either 300 mg of ESFAM or maltose as a placebo in the form of a capsule after breakfast.

Procedures

A randomized double-blind placebo-controlled trial (RCT) was conducted. The patients were randomly assigned into either ESFAM or placebo, through block randomization (four patients per block), supplementation was administered for eight weeks.

Peripheral blood samples were drawn from the patients through vein at the baseline and at the end of the study period. Venous blood samples used for the *ex vivo* study were centrifuged at 3,000 rpm for 10 minutes to obtain serum and then labeled and maintained at -80°C until analysis. *Ex vivo* study was performed by treating colorectal cell lines blindly with the serum of patients from both groups.

Best result of *ex vivo* study could be achieved by using treated patients cancer cells. Before using commercial cell lines from American Type Culture Collection (ATCC®), efforts to develop cell line derived from CRC patients were failed as the cells from patients underwent surgery was highly contaminated since these are colorectal tissues.

The human colorectal cell line types used in this study were COLO 205 and DLD-1 and were purchased from ATCC® (Catalog No. CCL-222 and Catalog No. CCL-221, respectively); they were maintained according to supplier guidelines (American Type Culture Collection, ATCC, Manassas, VA). DLD-1 is derived from the Dukes' type C human colorectal adenocarcinoma tissue,

and COLO 205 is derived from the Dukes' type D human colorectal adenocarcinoma tissue. Both lines were isolated from metastatic sites but there are differences, DLD-1 is from local metastatic site (lymph node) while COLO 205 is from a distant site (ascites). Both types of cell lines were chosen to represent both type of metastases.

Cells were incubated with 95% air and 5% CO₂ at 37°C; all cells were maintained below passage 20 and used in experiments during the linear phase of growth. The human colorectal cancer cell lines DLD-1 and COLO 205 were maintained in RPMI-1640 medium that supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin and maintained in a humidified environment containing 5% CO₂ at 37°C.

***In vitro* cytotoxic test by MTT assay**

Antiproliferative effect of water extract of *A. muricata* leaves and its fractions, dose range 62.5–2,000 µg/mL, were evaluated against colorectal cancer cell line DLD-1 and COLO 205 with micro-culture assay based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); compared with 5-fluorouracil (5-FU) and placebo.

The cytotoxic activity of extract was assessed using an MTT assay; cells were cultured in 96-well micro titer plates, where each well contained 2×10⁴ cells, and treated for 48 hours. Cytotoxicity was assessed using the MTT test (Trevigen's TACS® MTT cell proliferation assay), in triplicate.¹⁵⁻¹⁶

Caspase activity assessment

Since apoptosis might be one of the prime candidate mechanisms, we analyzed the expression level of caspase-8 and caspase-9. The DLD-1 colorectal carcinoma cell line was chosen since it was shown to be a proper *in vitro* model as previously described.¹⁷ Cleaved caspase-8 was measured using kit from R&D Systems, Inc. (USA & Canada) Catalog No. KCB705. This cell-based enzyme-linked immunosorbent assay (ELISA) contains the components required to run an ELISA using fluorogenic substrates to measure cleaved caspase-8 (Asp391) in whole cells.

This simple and efficient assay removes the need to prepare cell lysates and can be used to investigate

signaling pathways and the inhibitory property on cells. Cells were fixed and permeabilized in the wells after being grown in 96-well microplates and stimulated with ligands.

The target protein amount was assessed using a double immune enzymatic labeling procedure. Two primary antibodies were used to simultaneously incubate the cells. They are an antibody specific for the target protein and a normalization antibody that is specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping protein. The primary antibodies are obtained from different species. Two secondary antibodies recognizing the different species are labeled with either horseradish-peroxidase (HRP) or alkaline phosphatase (AP), and two spectrally distinct fluorogenic substrates for either HRP or AP are employed for detection. The fluorescence of the target protein is normalized to that of the total GAPDH in each well for the correction of well-to-well variations.

This two-wave length assay results in precise analysis of cleaved caspase-8 (Asp391) with good reproducibility. Cleaved caspase-9 was measured using kit from eBioscience company, Catalog No. BMS2025/BMS2025TEN (USA & Canada).

Concentrations of extracts used to test the caspase activity are 25–50 µg/mL, as compared to 25–50 µg/mL 5-FU as positive control. The extracts data were complemented by parallel studies of direct addition of participants' serum to the same human cell lines.

Ex vivo study was performed by treating colorectal cell lines with serum of patients from both groups; treated with leaves extract or placebo. Sera from patients of both groups were compared for caspase-8 and caspase-9 enhancement activity.

Statistical analysis

SPSS for Windows version 22 (SPSS Inc., USA) was used to analyze the results. The Saphiro Wilk test was used to test the normality of the data. Independent T tests was used to examine differences in means for normally distributed data, otherwise Mann Whitney U test was used. The p value was set at 0.05.

RESULTS

Cytotoxicity

Inhibitory concentration (IC)₅₀ values of the different extracts against DLD-1 are shown in Figure 1. ESFAM showed the lowest IC₅₀ values, 20.59 µg/mL.

Zirzak Orac and ESFAM showed the lower IC₅₀ values against COLO 205 compared to 5-FU which were 277.7 µg/mL and 654.9 µg/mL, respectively (Figure 2).

Caspase activity

The activity of caspase-8 and caspase-9 of DLD-1 colorectal cancer cell line tended to be higher when treated with the extracts as compared to 5-FU as positive control, as well as untreated

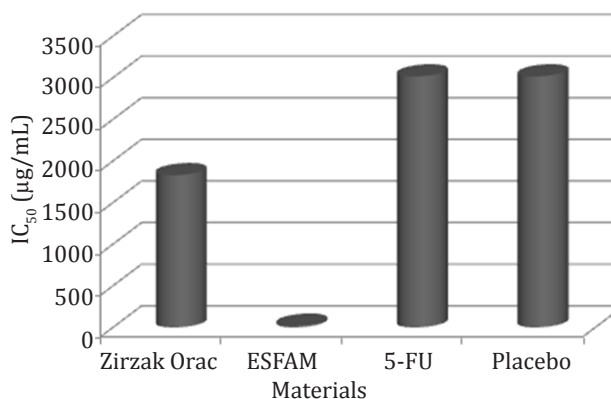


Figure 1. IC₅₀ of materials against DLD-1. ESFAM: ethanol-soluble fraction of *A. muricata* water extract; 5-FU: 5-fluorouracil

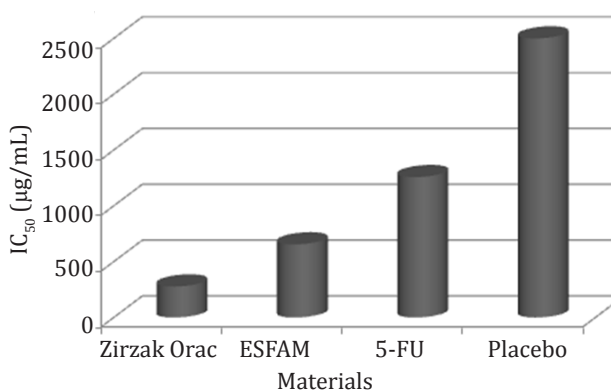


Figure 2. IC₅₀ of materials against COLO 205. ESFAM: ethanol-soluble fraction of *A. muricata* water extract; 5-FU: 5-fluorouracil

cell and blank that only contained medium (Figure 3).

Ex vivo experiment on caspases activities were conducted using subjects' serum to stimulate caspase-8 and caspase-9 activities of DLD-1 colorectal cell lines. The results showed that serum from patients treated with ESFAM significantly enhanced the expression of caspase-9 ($p < 0.05$) compared to placebo group. Serum from placebo group has significantly lower expression of caspase-9. Expression of caspase-8 tended to decrease in both groups ($p = 0.057$) (Table 1).

DISCUSSION

The cytotoxic activity of different extracts of *A. muricata* leaves have been assessed against human colorectal cancer cell lines COLO 205 and DLD-1. There was a dose dependent manner in decreasing viable cells after treatment with ESFAM and Zirzak Orac (0.0625–2 mg/ml) for 48 hours both in COLO 205 and DLD-1 cell lines. ESFAM showed the lowest IC_{50} against DLD-1, while Zirzak Orac and ESFAM had the lowest IC_{50} against COLO 205.

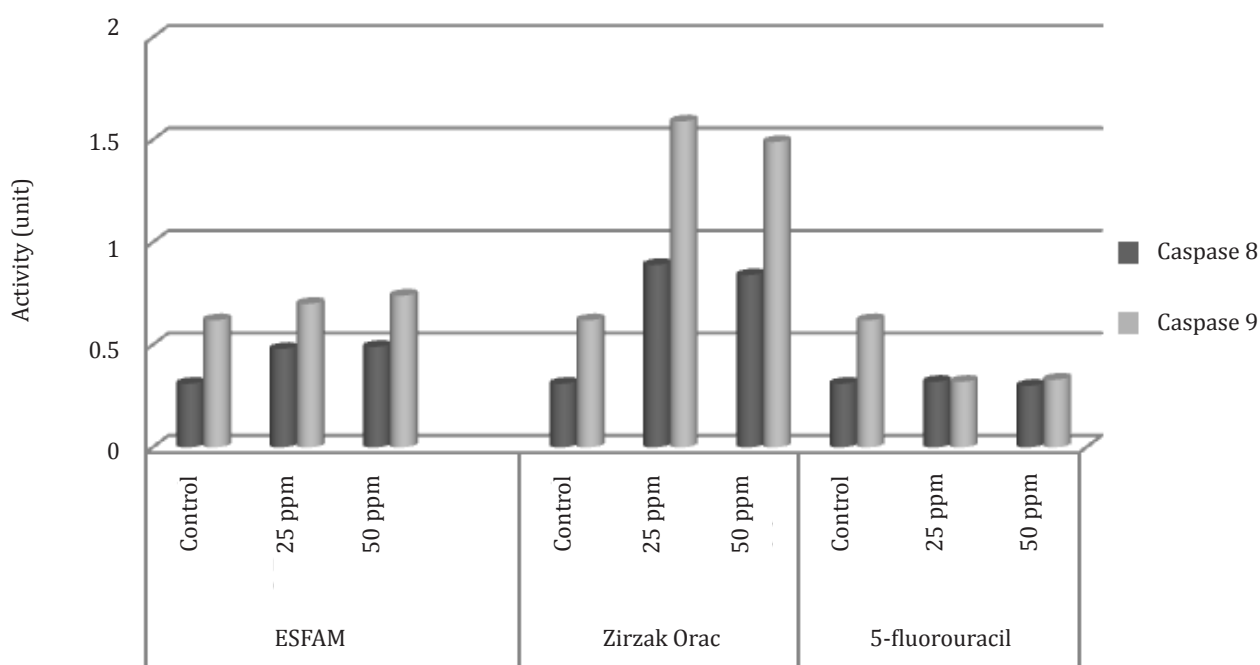


Figure 3. Caspase-8 and caspase-9 activities of DLD-1 colorectal cancer cell lines *in vitro*. ESFAM: ethanol-soluble fraction of *A. muricata* water extract

Table 1. Caspase-8 and caspase-9 activities of DLD-1 colorectal cell lines *ex vivo* before and after treatment

	ESFAM (unit) n=10	Within group difference (p value)	Placebo (unit) n=10	Within group difference (p value)	Between group difference (p value) ‡
Caspase-9					0.001
Before	0.59 (0.49–0.71)	0.048	0.62 (0.52–0.79)	0.014	
After	0.68 (0.60–0.75)		0.54 (0.47–0.64)		
Caspase-8					0.372
Before	0.17 (0.14–0.20)	0.057	0.19 (0.16–0.23)	0.433	
After	0.15 (0.13–0.19)		0.19 (0.16–0.23)		

*Data are; †median (25th, 75th percentiles); ‡Mann-Whitney U test was performed. ESFAM: ethanol-soluble fraction of *A. muricata* water extract

These findings are consistent with previous *in vitro* studies in demonstrating the cytotoxic activity of the leaves extracts, as well as its fractions, against colon adenocarcinoma (HT-29) cell lines, pancreatic carcinoma cell (PACA-2), prostate adenocarcinoma (PC-3), hepatoma cell lines Hep G2 clone 2,2,15 and HeLa cell culture and T47D breast cancer cell lines.¹⁸⁻¹⁹ Ethanolic extract of the leaves from Colombia, Indonesia, and Taiwan showed inhibitory activities against Cells-MDBK, CA-Mammary-MCF-7, and HepG2 clone 2,2,15 respectively. Ethanolic extract of the leaves from Borneo, Costa Rica, USA showed inhibitory activities against cell culture CA-9KB.¹¹ *In vivo* study in mice showed that annonacin inhibited the growth of the lung tumors during two-weeks period, it did not eradicate the tumors nor stop their growth in mice.

This study revealed the superiority of the extracts *in vitro* against colon cancer cells compared to 5-FU; and consistent with other studies showed that acetogenins as well as the extract are more potent than the standard drugs (adriamycin, vincristine, and vinblastine).¹⁰⁻¹¹ Data from previous study on colon cancer cell lines suggested that there were pathways for 5-FU resistance including altered regulation of nucleotide metabolism, amino acid metabolism, cytoskeleton organization, transport, and oxygen metabolism.²⁰ The cytotoxicity of acetogenins has been shown to be stronger in tumorous than in normal cells.⁹ This study showed that ESFAM with the higher concentration of annonacin showed similar cytotoxicity against both types of cell lines.

Study on caspase activity was conducted to determine the mechanisms of action of the cytotoxicity of *A. muricata* leaves extracts. Caspase-8 and caspase-9 activities were tested both *in vitro* and *ex vivo* on human colorectal cancer DLD-1 cell lines. Caspase-8²¹ and caspase-9¹⁷ were the most important regulator in DLD-1.

The activities of both caspases tend to increase by induction of ESFAM and Zirzak Orac compared to 5-FU and control cell *in vitro*. These results are in agreement with previous studies, where ethanolic extract of *A. muricata* induced apoptosis in the myelogenous leukemic K562 cell line through inducing caspase-3 activity and deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL).²²

This study was the first *ex vivo* study for *A. muricata* leaves extract that exposing CRC cell lines with patients serum after eight weeks treatment with ESFAM. The use of serum from patients supplemented with ESFAM was aimed to overcome the limitation in conducting bioavailability study, which was not currently possible due to uncertainty of bioactive compounds in the extract. *In vitro* study on ESFAM may reflect the effect of the extract against the cell tumor directly when it passed through the tumor in colon, and *ex vivo* study using patients' sera represented the effect of ESFAM after being absorbed and entered the blood circulation.

The result of *ex vivo* caspase activity study is consistent with the *in vitro* study. Patients' serum from ESFAM group significantly increased the activity of caspase-9 compared to placebo group. On the contrary, patients' serum from placebo group tended to decrease caspase-9 activity.

Annonaceous acetogenin has been shown previously inducing apoptosis in cancer cell lines by involving multiple pathways. The primary site of action of the acetogenins is complex I of the electron transport chain in mitochondria.¹⁰ Acetogenins have also been shown being the potent inhibitors of NADH oxidase in plasma membranes of cancerous liver cells. Specifically, the bis-THF acetogenin bullatacin was shown to be a strong inhibitor of this enzyme in liver cancer cell; however, bullatacin showed no inhibition of the NADH oxidase against "normalized" liver cells.²³

The enhancement of caspase-9 activity in this study is consistent with the above studies where caspase-9 plays key role in intrinsic pathway, namely mitochondrial-mediated pathway. Patients' serum induces cytotoxicity after consumption of *A. muricata* leaves extract most probably mediated through the effect of the extract in enhancing caspase-9 activity.

Activity of caspase-8 in *ex vivo* study was contradictory with result of *in vitro* study, where caspase-8 activity tended to decrease instead of increase. This result may indicate that the pathway for apoptosis capacity is not through extrinsic pathway. The non-significant result may also due to inadequate dose given to subjects, resulted in an insufficient amount in the serum

to enhance the caspase-8 activity. It may also due to low concentration of serum in the cell culture media, which is only 10%. Number of caspase-8 reseptor on DLD-1 cell lines may also contribute to this result.

In conclusion, *A. muricata* leaves water extract and its ESFAM exhibited cytotoxic activities in colorectal cancer cells *in vitro*. ESFAM significantly induced caspase-9 activity of DLD-1 colorectal cancer cell line *ex vivo* using serum of colorectal cancer patients after eight weeks treatment with ESFAM.

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Conflicts of interest

The authors affirm no conflict of interest in this study.

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