Original Article

The effect of an *Annona muricata* leaf extract on nutritional status and cytotoxicity in colorectal cancer: a randomized controlled trial

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Background and Objectives: *Annona muricata* leaf infusion has traditionally been consumed to maintain health, but is now considered for use in treating cancer patients. The objective of this study was to elucidate the effects of *A. muricata* leaf extract in humans and human cell lines. **Methods and Study Design:** Thirty outpatients with colorectal cancer who had undergone primary tumor resection were enrolled in a randomized double-blind placebo-controlled pre–post-trial. They were divided into two groups: those who ingested *A. muricata* leaf extract (n=14) and those who ingested a placebo (n=14) daily for 8 weeks. Twenty-eight subjects completed the trial; they were equally distributed between the two groups. Serum from patients of both groups was compared for cytotoxicity against colorectal cancer cell lines. The nutritional status of patients was monitored throughout the study. **Results:** *Ex vivo* and clinical studies showed higher cytotoxicity in the supplemented group compared with the placebo group. Further research is required to investigate the long-term effect of *A.muricata* leaf extract, particularly on parameters directly related to cytotoxic activity toward colorectal cancer cells and nutrition status.

Key Words: ***Annona muricata*, leaf extract, nutrition status, colorectal cancer, cytotoxicity**

INTRODUCTION

The prevalence of colorectal cancer is rising in Asia[1], where it is now the third most common malignant disease in both men and women. Data from the International Agency for Research shows that the incidence of colorectal cancer in many affluent Asian countries is similar to that in the West. In recent decades, Eastern Asian countries such as China, Japan, South Korea, and Singapore have experienced a two-to-four fold increase in the incidence of this disease,[2] and data from 13 cancer registries in Indonesia show that colorectal cancer is one of the five most prevalent cancers in males and females.[3]

Annona muricataL. (Soursop) belongs to the Annonaceae family. It has been investigated as a potential source of biologically active annonaceousacetogenins, some of which have demonstrated powerful antitumor activity.[4] The cytotoxicity of acetogenins was found to be stronger in tumorous cells than in normal cells.[5] One study showed that application of an ethyl acetate extract of *A. muricata* leaf (EEAML) significantly reduced the formation of colonic aberrant crypt foci (ACF) compared with that in a cancer control group. When isolated from EEAML, annomuricin E inhibited the growth of HT-29 cells with an IC50 value of 1.62±0.24 μg/mL after 48 h. The cytotoxic effect of annomuricin E was further substantiated by G1 cell cycle arrest and early apoptosis induction in HT-29 cells. Annomuricin E triggered mitochondria-initiated events, including the dissipation of the mitochondrial membrane potential and leakage of cytochrome c from the mitochondria. Prior to these events, annomuricin E activated caspase 3/7 and caspase 9, and upstream it induced time-dependent upregulation of Bax and downregulation of Bcl-2 at the mRNA and protein levels.[6]

The nutrition status of patients with gastrointestinal cancer is related to both a systemic inflammatory response and an advanced stage of disease. Results of an animal study have suggested that ethanolic extract of *A. muricata* leaves can be considered a potential source of bioactive compounds with antinociceptive and anti-inflammatory activity, such as alkaloids, essential oils, and acetogenins.[7] The extract was observed to be effect-

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ive in overcoming acute and chronic inflammation,[8] and its antioxidative properties were reported.[9]

The reported evidence motivated this human study, which explored the effect of *A. muricata* on *ex vivo* parameters and evaluated its effect on colorectal cancer through an assessment of nutrition status.

METHODS

Subjects and trial criteria

The subjects were colorectal cancer (CRC) outpatients at the CiptoMangunkusumo teaching hospital, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia, after having undergone surgery for complete primary tumor resection. Thirty patients were recruited and randomly assigned to either the *A. muricata* leaf extract group or to a placebo group for 8 weeks. The study protocol was approved by Ethics Committee, Faculty of Medicine, University of Indonesia (406/H2.F1/ETIK/2013), and registered on ClinicalTrials.gov under the identifier NCT02439580. Written informed consent was obtained prior to the study, with voluntary participation.

Inclusion criteria

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 therapeutic treatment throughout the study period were included in the study. In addition, the patients were required to have satisfactory hematological and biochemical function and a Karnofsky performance status of ≥60%.

Exclusion criteria

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; kidney, liver, endocrine, or neurologic or psychiatric disease; a disability rendering them unable to communicate verbally; or a history of cancers other than colorectal (such as nonmelanoma skin cancer, basal cell carcinoma, and squamous cell carcinoma) in the past 5 years. Pregnant or lactating women, and those not using adequate contraception, were also excluded. In addition, patients taking other investigational drugs, patients with HNPCC (Lynch syndrome), 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 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.

Protocol

A randomized double-blind placebo-controlled pre–post trial (RCT) was conducted. The patients were assigned consecutively into either ESFAM or placebo, through block randomization (four patients per block); supplementation was administered for 8 weeks.

Anthropometry and dietary intake measurements were conducted every 2 weeks throughout the study period (Figure 1). The patients recorded their food intake as measured using a food scale, and energy and nutrient intakes were calculated using average values calculated over 2 days.

Peripheral blood samples were drawn from the patients through venipuncture at the baseline and at the end of the study period; the hemoglobin level was analyzed using an automated biochemical analyzer (Sysmex).

Ex vivo study

Venous blood samples used in ex vivo study were centrifuged at 3,000 rpm for 10 min to obtain serum and then labeled and maintained at -80°C until analysis. Ex vivo study was performed by treating colorectal cell lines with the serum of patients from both groups. The cytotoxic activity of serum was assessed using an MTT assay; cells were cultured in 96-well microtiter plates, where each well contained 2×10^4 cells, and treated for 48 h. Cytotoxicity was assessed using the MTT test (Trevigen's TACS® MTT Cell Proliferation Assay), in triplicate.[10-11]

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). In addition, the human embryonic kidney (HEK) cell line was used as the normal control cell line; it were obtained from the Institute of Human Virology and Cancer Biology, University of Indonesia.

DLD-1 is derived from the Dukes' type C human colorectal adenocarcinoma tissue of a male subject, and COLO 205 is derived from the Dukes' type D human colorectal adenocarcinoma tissue of a 70 year old Caucasian male. Both lines were isolated from metastatic sites. Cells were incubated with 95% air and 5% CO^2 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 RPMI1640 medium and HEK was maintained in DMEM, both mediums were supplemented with 10% FCS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin and maintained in a humidified environment containing 5% CO^2 at 37°C.

Statistical analysis

Analysis was conducted using SPSS for Windows software version 22 (SPSS Inc., USA). Clinical trial data on patient characteristics were analyzed using descriptive statistical analyses, and independent t tests were used to examine differences in means for normally distributed continuous variables (age, weight, BMI). The paired sample t test was used to examine differences in means within groups for normally distributed continuous variables. Non-normally distributed data were examined through nonparametric tests, and the Saphiro Wilk test was used to test normality of data. The general linear model was used to analyze repeated measurements of

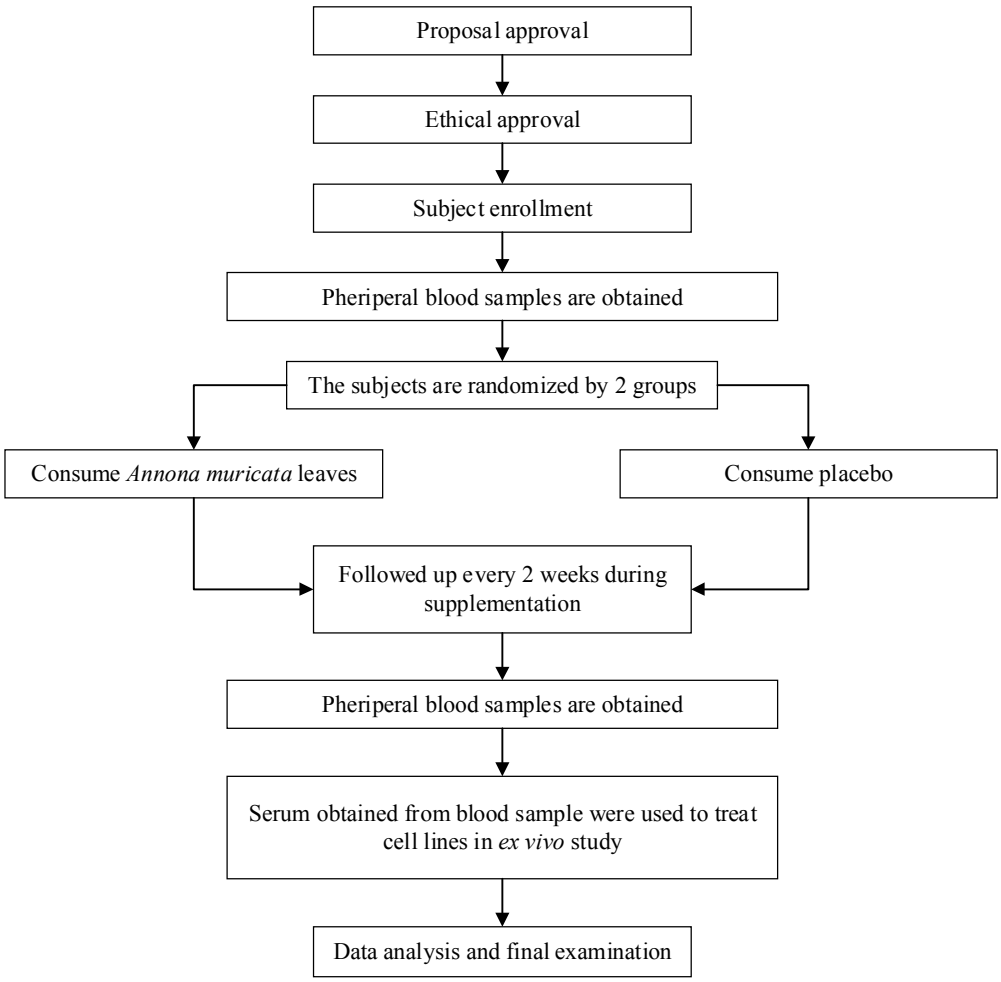

Figure 1. Flow chart of study design

nutritional status during supplementation. The *p* value was set at 0.05 (5%).

RESULTS

Nutrient intakes

Of 253 patients from the two centers, 30 met the inclusion criteria and consented to take part in the trial; they were randomly allocated into two groups (n=15), those taking an ESFAM supplement and those taking a placebo (control group). Although 30 patients participated, only 28 aged 30–80 years (x=50.2 years) completed the study, and this number was divided equally between the placebo (n=14) and the ESFAM (n=14) group. The baseline characteristics of the patients are shown in Table 1. Table 2 shows the nutrition status and hemoglobin concentration of the patients at the baseline, which was comparable between the groups.

Ex vivo experiment

The ex vivo experiment on cytotoxic activity was conducted using serum from the CRC patients. Table 3 shows significant growth inhibition of DLD-1 colorectal cancer cells line (p=0.013) after ESFAM administration compared with before ESFAM administration (baseline).

Serum from the ESFAM group also showed cytotoxicity against COLO 205 (p=0.08) compared with the baseline serum, but there was no significant difference compared with the placebo group. In consideration of the aforementioned results, a value of $p \leq 0.08$ can be used instead of $p<0.05$ to show a significant difference be-

Table 1. Medical history of patients in each group

		Groups		Between group difference *p* value[†]
		ESFAM (n=150)	Placebo (n=15)	
Gender (%)	Men	66.7	66.7	1.00
	Women	33.3	33.3	
Age (yr)		51.0±15.0	52.2±10.2	0.79
Chemotherapy (%)	Received	80.0	73.3	1.00
	Not	20.0	26.7	
Tumor stage (%)	I-II	26.7	33.3	1.00
	III-IV	73.3	66.7	

[†]Chi-square test was performed.

Table 2. Nutrition status and haemoglobin levels of patients at the baseline

Nutrition status	Groups†		Between group difference
	ESFAM (n=15)	Placebo (n=15)	*p* value‡
Total energy intake (kcal/day)	1788.9±494.1	1487.6±564.5	0.14
BMI	23.3±0.0	21.0±3.2	0.17
Hemoglobin	12.7±1.6	12.6±1.5	0.88

†Data are expressed as the mean±SD.
‡Independent sample t test was performed.

Table 3. Cytotoxicity of serum against cell lines in each group

		ESFAM (n=10) Living cells (%)	Within group difference *p* value	Placebo (n=10) Living cells (%)	Within group difference *p* value	Between group difference *p* value‡
HEK	Before	70.46 (58.33-78.27)	0.08	71.1 (58.4-98.2)	0.04	0.006
	After	78.63 (64.32-91.29)		66.0 (58.5-71.7)		
DLD-1	Before	56.28 (33.54-105.06)	0.01	32.7 (15.6-93.6)	0.86	0.08
	After	52.16 (36.70-74.92)		49.7 (33.3 -71.7)		
COLO 205	Before	83.5±29.81	0.08	81.6 (66.6-130.1)	0.09	0.47
	After	72.28±24.03		72.7 (57.5-88.4)		

†Data are expressed as the mean ± SD for normally distributed data and the median (25th, 75th percentiles) for non-normally distributed data.
‡Independent sample *t* test was performed for normally distributed data, Mann-Whitney U test for non-normally distributed data.

tween groups.

The serum obtained from the ESFAM group significantly increased HEK cells (p=0.006) compared with the serum from the placebo group, which showed significant cytotoxic activity against HEK cells compared with the baseline (p=0.04). HEK cells are normal cells, hence, serum was not expected to have cytotoxic activity.

Nutrient intake and anthropometry

In general, the total energy intake (Figure 2) of the patients was lower than the recommended daily allowance for Indonesia (Permenkes No.75, 2013) in all age groups. There was no significant difference in nutrient intake between groups throughout the 8 weeks indicating that supplementation had no effect on the energy intake of either the ESFAM group or the placebo group. However, there was significant increase within group as time point. In the ESFAM group, energy intake at week 8 (1787.55±620.2 kcal) significantly increased compared with week 4 (1544.31±560.43 kcal) and week 6 (1596.6±531.11 kcal), at p=0.04 and p=0.02, respectively. In placebo group, there was no significant differences in energy intake throughout the 8 weeks of measurement, and the energy intake was stable at all points of observation.

There was also no significant difference in the anthropometric status between groups. Comparing the endline values of both groups with the baseline values revealed that the nutrition status of both groups was maintained. In addition, the BMI of the patients during supplementation was stable among the measurement points, with the BMI difference being lower than one unit between the groups throughout the 8 weeks (Figure 3). Furthermore, hemoglobin concentrations before and after supplementation were within a normal range of values, showing that ESFAM did not reduce the hemoglobin concentration (Table 4).

Combining human and ex vivo studies

Nutrition status and hemoglobin concentrations are considered as quality of life indicators. Data obtained from

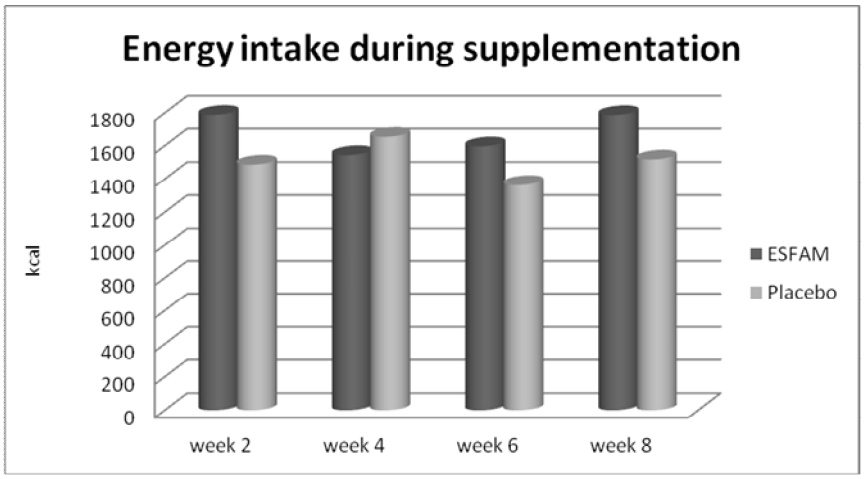

Figure 2. Energy intake during trial

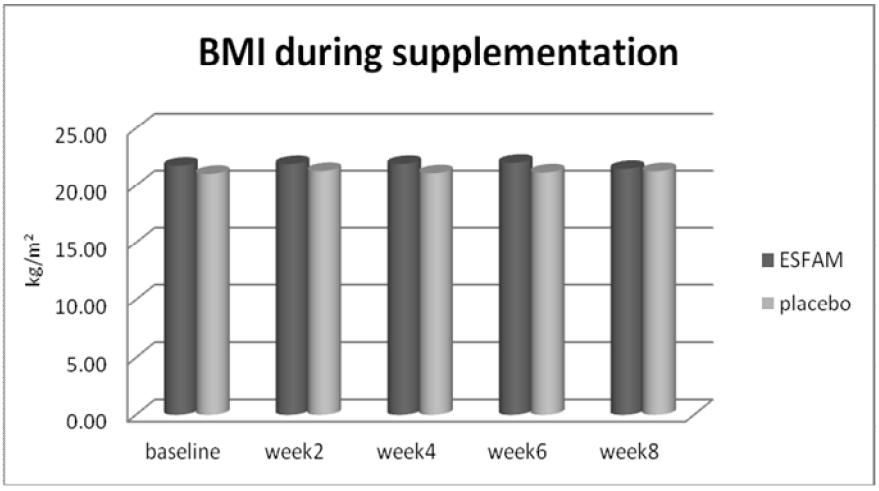

Figure 3. Changes in BMI throughout trial

Table 4. Haemoglobin concentrations in each group

	Baseline	Endline	Within-group difference *p*-value	Between-group difference *p*-value‡
ESFAM (n=15)	12.7 ±1.7	12.8±2.1	0.582	0.867
Control (n=15)	12.4±1.4	12.6±1.7	0.539	

†Data are expressed as the mean±SD.
‡independent sample t test was performed.

Table 5. Combined human and *ex vivo* studies

Group	Energy intake stable/increased	BMI stable/increased	Hemoglobin stable/increased	HEK (normal cell) increased	DLD-1 or COLO 205 (CRC cell) decreased
ESFAM	66.7%	91.7%	100%	72%	97%
Placebo	66.7%	91.7%	100%	14.3%	58.3%

the ESFAM group revealed that energy intake, BMI, and hemoglobin concentrations remained stable or increased (Table 5). Moreover, the results from the placebo group were comparable (Table 5), meaning that the quality of life of the patients in both the ESFAM and placebo groups was maintained throughout the 8 weeks as shown by stable or increased energy intake and BMI. Ninety seven percent of the serum obtained from ESFAM group exhibited an inhibitory property against one or all of the CRC cell lines. By contrast, only 58.3% of the serum in placebo group showed inhibitory properties against one or all of the CRC cell lines (Table 5). While 72% of the serum obtained from ESFAM group increased normal HEK cells and only 14.3% of the serum in placebo group showed an ability to increase normal HEK cells (Table 5).

DISCUSSION

This study is the first to expose CRC cell lines to the serum of patients extracted after supplementation with ESFAM. Serum from patients supplemented with ESFAM was used because it currently is not possible to conduct bioavailability studies as a result of the uncertainty regarding the bioactive compounds in the extract. *In vitro* study on ESFAM may reflect the direct effect of the extract on tumor cells as it passes through tumors in the colon; *ex vivo* study using patient's serum is considered to represent the effect of ESFAM after it is absorbed and reaches the plasma. In non-gastrointestinal cancer, the amount of extract that reaches the blood (bioavailability) is the fraction considered able to exert an effect on cancer cells.

The previous *in vitro* cytotoxicity against colorectal cancer cell lines[12] are in agreement with the present *ex vivo* study, indicating that the bioavailability of the extract was adequate, enabling it to reach the blood and inhibit cancer cells. The results of this *ex vivo* study show that the serum had a higher inhibition capacity toward DLD-1, which may be due to the difference in cell line origins: DLD-1 is derived from Dukes' type C, whereas COLO 205 is derived from Dukes' type D colorectal adenocarcinoma, which is a more advanced stage colorectal cancer than Dukes' type C. Before using cell lines derived from ATCC, efforts to develop cell line derived from CRC patients (primary tumor cell line) was highly contaminated since these are colorectal tissue.

The results of the present study also showed that serum from the ESFAM group exhibited selectivity in exclusively inhibiting the growth of colorectal cancer cells and not inhibiting the normal cells. The cytotoxicity of acetogenins was reported to be stronger in tumorous cells than in normal cells:[5] The primary site of action of acetogenins is complex I of the electron transport chain in mitochondria.[13]

Another study evaluated the chemopreventive properties of EEAML on azoxymethane-induced colonic ACF in rats. The cytotoxic compound of EEAML (annomuri-

cin E) was isolated, and its apoptosis-inducing effect was investigated against the HT-29 colon cancer cell line.[6] The cytotoxic activity in this in vitro study was consistent with the present *ex vivo* study, implying that active ingredients were able to reach the blood and inhibit the colorectal cancer cells in the present study.

The patients in this study were asked to record their food intake, mainly to assess their appetites. The inhibition of tumorous cells was expected to improve the general condition of the patients, including their appetite and nutrition status. However, no significant changes in the food intake or anthropometry data at any of the four measurement points were observed between the groups, indicating that the nutrition status of patients in both groups was stable throughout the 8 weeks of supplementation. However, there was a significant energy intake increase ESFAM group but this improvement was not observed in the placebo group. Longer supplementation period may improve the nutrition status of patients, and a larger and longer cohort study is required to test this hypothesis.

Previous studies have suggested that nutrition is a major concern in oncology, and a decline in nutrition status may ensue from both the course of the disease and its treatment. Cancer-associated malnutrition and a progressive loss of body weight are common features in most cancer patients, although prevalence rates vary from9% to 85% depending on the tumor type and disease stage.[14]

Some patients in this study took the extract in parallel with chemotherapy. However, their compliance remained acceptable despite the adverse effects of chemotherapy, and there was neither bodyweight loss nor a reduction in nutrition intake during supplementation of *A. muricata* extract, confirming there was no detrimental effect.

The most common adverse effects of chemotherapy on nutrition status are anorexia, altered perception of taste and smell, food aversion, nausea and vomiting, mucositis, xerostomia, constipation, diarrhea, and early satiety. Chemotherapy may also cause abdominal cramps and bloating, paralytic ileus, and even malabsorption. Some antineoplastic agents such as fluorouracil, adriamycin, methotrexate, and cisplatin may induce severe gastrointestinal complications.[14] In this study, some of the patients undergoing chemotherapy felt better while consuming the *A. muricata* leaves extract supplement, and one subject felt that the mass in her abdomen was less uncomfortable and she experienced reduced stimulation to strain.

Many findings on phytochemicals are inconclusive because the studies are conducted either in vitro or by using animals *in vivo*. This is partly due to our limited understanding on phytochemical bioavailability, on which health benefits depend. In addition, the transport mechanisms for delivering phytochemicals to target sites, the phytochemical metabolism of the human body, and biomarkers exerting health benefits are poorly understood. Using subjects's serum who have ingested the *A. muricata* leaves extract to examine the effect of its bioactive compounds on cells is a more suitable method in elucidating the effect of the bioactive compounds on the human body than directly treating cells in vitro by the extract. This approach is more relevant, because bioavailability data on phytochemicals in vivo are limited.

The present RCT provides evidence that serum from patients taking the extract showed cytotoxicity against human colorectal cancer cell lines. The reduced viability of cancer cells may improve the nutrition status and quality of life of cancer patients, revealed that an adequate amount of extract reaches the blood to kill cancer cells. Therefore, an optimal dose should be determined for administration to provide the adequate amount required to reach the blood. However, the present RCT cannot be expected to produce results that are directly relevant to all patients in all settings.

More importantly, the effect of *A. muricata* leaf extract should be studied in relation to the outcomes of various cancers over a study period longer than the human cell lifespan. For example, to examine the effect on red blood cell counts, a study should be conducted over a period of at least 120 days; the longer the study period, the more cells will benefit from the treatment, which may then improve the quality of life. Moreover, future studies should be performed using a more comprehensive study design.

More extensive studies are also required on the absorption and bioavailability of the extract and its fractions. In addition, most dietary compounds contain a mixture of acetogenins and other bioactive compounds may present, and these should be identified in *A. muricata* extract. Some phytochemicals may have synergistic effects, although they are not individually effective as chemopreventative agents. Thus other bioactive compounds, in addition to acetogenins, should also be explored to provide a thorough understanding on the chemopreventive effect of the extract and its fractions. Additional extensive studies are thus required.

Finally, the amount of extract used in this study, in terms of the equivalent concentration of the active ingredient, was extrapolated from the amount traditionally consumed. This was performed to find an effective and affordable treatment for cancer patients. Thus, the amount of *A. muricata* used traditionally, which is in line with the results of *in vitro*, *ex vivo*, and pilot human studies, can be used as an adjunct to standard treatment for colorectal cancer patients at a dose of 7–15 cups daily prepared as an infusion which is equivalent to 1030 mg annonacin. However, a study with a large cohort of colorectal cancer patients and a thorough evaluation of the efficacy and safety of each fraction of the water extract is required to confirm this finding and recalculate the dose.

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AUTHOR DISCLOSURES

The authors have no financial or commercial conflicts of interest in this work.

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