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RESEARCH ARTICLE

Inflammatory Response of Annona *Muricata Linn* Leaves Extract in Colorectal Cancer Patients

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Abstract

Annona muricata L (AML) is a member of Annonaceae with antioxidant, anti-inflammatory and anticancer properties. A randomized double blind placebo controlled pre post-trial on twenty colorectal cancer patients who had undergone primary tumor resection were conducted in two groups, each group was supplemented with *A. muricata* leaves extract, and maltose as placebo. Inflammatory responses were assessed using ELISA method. There is no significant difference between pre and post on cytokine and cyclooxygenase serum level, also between two groups. A strong significant correlation between serum level of TNF-α and IL-10 cytokine (coefficient correlation = 0.64, p = 0.01) and significant correlation between IFN-γ and IL-10 (coefficient correlation = 0.47, p = 0.05) was found after 8 weeks AML supplementation, as shown by increasing of IL-10 production, in response to TNF-α and IFN-γ in an inflammatory condition. This study is registered in Clinical Trials.gov ID: NCT02439580

Keywords: *Annona muricata L, inflammatory response, colorectal cancer, cytokine, cyclooxygenase, TNF-α, Interleukin-10, Acetogenin.*

Introduction

Cancer is the second most common cause of death in the US, while the incidence rates continue increasing.[1] Colorectal cancer is one of the most common causes of cancer death after lung and breast cancers in women, or prostate in men.[2] The prevalence of colorectal cancer is increasing and it is now the third most common malignant disease in both men and women in Asia.[3] In Indonesia, colorectal cancer is one of the five prevalent cancer among 13 cancer registries found.[4] Two main risk factors of colorectal cancer are hereditary factor and inflammation. The longer the duration of inflammation, the greater the risk of developing colorectal cancer. In the inflammatory process, the cells involved (neutrophils, monocytes, macrophages and eosinophils) produce soluble factors such as arachidonic acid metabolites, cytokines, chemokines, and free radicals which mediated cancer development. Chronic exposure to these mediators causes the increase of cell proliferation, mutagenesis, oncogene activation and angiogenesis which in turn cells will be unabled to control growth.[5]

The main treatment for most primary colorectal cancer is surgery which aimed to resect the cancer tissue and surrounding healthy tissues, followed by chemotherapy, radiotherapy and/or immunotherapy adjuvant,[6] even though it still has possibilities to recur. Mostly, the cancer diagnosed at an advanced stage so that cancer resection cannot be done completely. All the treatments above also affect the quality of life of colorectal cancer patients.

Hence, inexpensive treatments which could be well tolerated by the patients need to be explored. Research on herbal medicines that

exhibit a potent anticancer activity shows many promising results.

Annona muricata Linn. (AML), or soursop is a family of Annonaceae with one of its active compound, acetogenin, reported to have cytotoxic effects. Acetogenin is a potent inhibitor of the mitochondrial NADH-ubiquinone reductase (Complex 1), and it induces apoptosis by reducing cAMP and cGMP levels in human cancer cells.[7] Besides the cytotoxic mechanism, AML leaf extract was reported to have anti-inflammatory function.[8]

Most of the studies about anti-inflammatory properties of AML leaves were in animal model. Hence, it is important to find out the role in the pro-inflammatory and anti-inflammatory balance regulation in colorectal cancer patients.

The inflammation-related colorectal carcinogenesis is a complex process which supports the neoplastic changes through gene mutations, cell proliferation, adaptation, angiogenesis and inhibition of apoptosis. Damaged DNA particularly in tumor suppressor gene and oncogenes will increase the risk of advanced neoplastic process. 5 Some inflammatory mediators such as prostaglandin and cytokine also play roles in cancer development. Prostaglandins, such as prostaglandin E (PGE 2) are derived from metabolism of arachidonic acid which catalyzed by the enzyme cyclooxygenase (COX). Increasing PGE2 specifically obtained in colorectal cancer[9] stimulates cell proliferation, angiogenesis induction, apoptosis inhibition, and acts as an immunosuppressive agent which weakened surveillance immune cells, hence it is unable to detect tumor.[5]

The cytokines promote proliferation of neoplastic cells in the early stages of tumor development, also involved in the production of free radicals. Cytokines profile in serum or in tumor can be a useful indicator for neoplastic changes and inflammation process.[10,11] Serum level of interleukin-2 (IL-2) was reported lower in individual with colorectal adenoma, while levels of IL-8, IL -10, IL-12, granulocyte macrophage colony stimulating factor (GMCSF), IFN-γ and TNF-α were higher, compared to control.[12] AML leaf extract has potent antioxidant activity,[13,14] antitumor, anti-cancer and selective toxicity against several types of cancer cells. As an anti-inflammatory substance, AML leaf decoction is traditionally used for compressing swollen feet.[13]

The ethanolic extract of AML leaf could inhibit prostaglandin synthesis, especially in higher dose,[15] and could reduce edema in animal inflammatory-mode.[16] Another study found that at higher dose, AML ethanol extract significantly reduced levels of the pro-inflammatory cytokines TNF-α and IL-1ß at sites of inflammation in animal model.[8] AML is also known to have immunomodulatory effects, increasing the number of CD4+ T and CD8+ T cell counts in the thymus significantly, at a dose of 25mg /kg.[17] The effect of AML leaves extract after supplemented to the CRC patients would like to be observed directly.

Materials and Methods

AML Preparation

The AML extract used in this study was a standardized vacuum dried extract (Zirzak Orac) of Javaplant, Central Java, Indonesia, containing 0.018% acetogenin (w/w).AML leaves aqueous extract was fractionated using 95% ethanol to produce ethanol soluble fraction (ESFAM) and ethanol insoluble fractions (EIFAM). ESFAM was supplemented to the subject. Acetogenin content of the extract was determined by thin layer chromatography (HPTLC) at the Faculty of Pharmacy, University of Gadjah Mada, Yogyakarta, Indonesia. ESFAM contains 0.36% w/w acetogenin or 3.6 mg/g.
In this study, 300mg ESFAM or maltose (as placebo) was given in the form of capsule, consumed by the subjects 1 capsule/day after breakfast.[18]

Study Population

Subjects in this study were post-resection colorectal cancer out-patient at Cipto Mangunkusumo Teaching Hospital, Faculty of Medicine, Jakarta, Indonesia, undergone standard therapy (surgery, chemotherapy or radiotherapy). Male and female CRC outpatient older than 30 years who were voluntary willing to administered one capsule of ESFAM or placebo per day in addition to medical treatment during eight weeks study period, with Karnofsky Performance Scale Index ≥ 60% were included in this study after signing informed consent.[19] Subjects with

history of heart, kidney, liver, endocrine disease, serious neurologic or psychiatric condition, uncontrolled hypertension and diabetes mellitus, serious heart, liver and kidney problem, physical disorder or limitations to communicate verbally or social interaction, familial adenomatous polyposis or other cancer in the last five years were excluded. Likewise, smokers, pregnant or breastfeeding women, under medication or supplements to other studies were also excluded.

Study Ethics

This study was a part in a clnical trial study of *Annona muricata* Linn. leaves extract supplementation to colorectal cancer patients[19] which approved and supervised by the Medical Ethics Committee, Faculty of Medicine, University of Indonesia, Indonesia (No. 406/H2.F1/ETIK/2013). The study and possible outcomes were explained to all participants and written informed consent were obtained from patients and their accompanying family members. This study is registered in Clinical Trials.gov ID: NCT02439580.

Study Protocol

A Randomized double blind placebo controlled pre-post trial was conducted on CRC out patients. Out of 253 CRC out patients recruited, 30 subjects were included and divided into 2 groups (n=15), ESFAM supplementation and maltose as placebo, respectively, 1 capsule of 300 mg/day for eight weeks.[19]

Anthropometry measurement was conducted and food intake was recorded in every 2 weeks throughout the study period by using a food scale. Clinical history data such as ages, sex, colorectal cancer stage, treatment were obtained from medical record.

Peripheral blood samples were routinely drawn through venipuncture in the morning following the standardized procedures.at the pre-admission before the commencement of initial treatment and at the end of the study. Venous blood samples were centrifuged at 3,000 rpm for 10 min to obtain serum, labeled and maintained at -80°C until analysis. Circulation level of TNF-α, IL-10, IFN-γ and COX-2 were measured using ELISA kit at Integrated Laboratory, Faculty of Medicine - University of Indonesia.

Outcome Measures

The concentration of TNF-α, IFN-γ, and interleukin-10 (IL-10) were assessed using Quantikine ELISA kits for TNF-α, IL-10 and IFN-γ human (R & D Systems, Inc., Minneapolis, USA). The lowest detectable dose was 1.6 pg/mL for TNF-α, 3.9 pg/ml for IL-10 and 8 pg/mL for IFN-γ. ELISA kit for Prostaglandin Endoperoxsidase Synthase 2 (PTGS2) (Cloud-Clone Corp,, Katy, USA). Was used for COX-2 level serum examination according to the manufacturers' instruction. Duplicates were not performed due to the limited number of blood samples. The result was read using Vmax Microplate Reader (Molecular Devices LLC, Sunnyvale, USA).

Statistical Analysis

Statistical analysis was conducted using SPSS for Windows software version 17 (SPSS Inc., USA). Independent t-test or paired t-test were used to examine mean differences for normally distributed continuous variable. Non normally-distributed data were examined using Mann-Whitney or Wilcoxon test. The p-value was set at 0.05 (95%).

Results

Study Population

A total of 30 subjects met the inclusion criteria from 253 patients, consented to participate in the study and randomly allocated into two groups (n = 15), ESFAM and placebo groups. Only 25 subjects completed the eight weeks supplementation, 13 subjects and 12 subjects of ESFAM and placebo group, respectively. There were 25 blood specimen of the subjects for cytokine examination, and 20 blood specimen of the subjects for COX-2 test due to limited blood sample, representing 10 subjects of each group (Figure 1). The study population consisted of 17 men (68%) and 8 women (32%) with an average age of 52.1 years (Table 1). There is no significant age and gender differences between the two groups ($p = 0.83$), and ($p = 0.61$), respectively. There are also no significant differences in cancer stage, chemotherapy, radiotherapy status, and BMI between two groups ($p>0.05$).

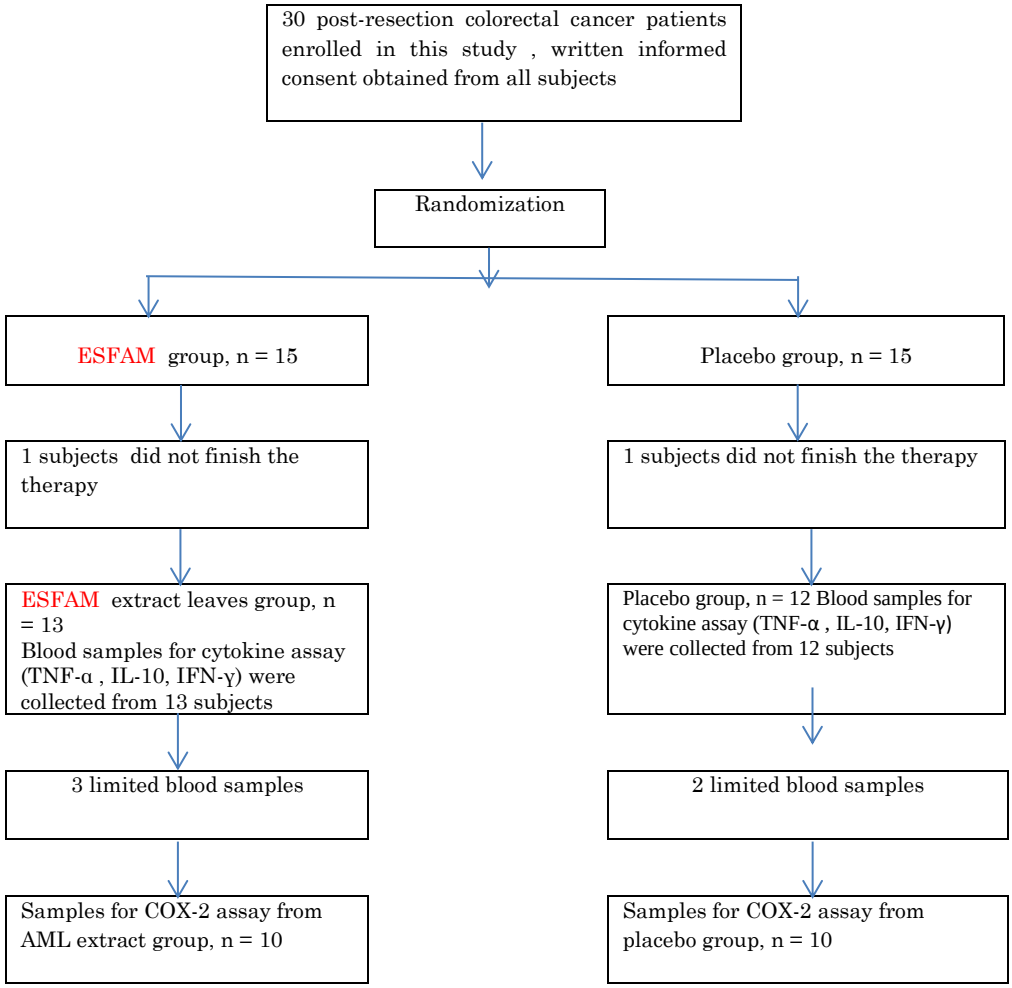

Fig. 1: Flow diagram for enrollment of subjects

Tolerability

None of the subject reported difficulty in tolerating oral intake of ESFAM at the applied doses.

Safety

Neither morbidity nor mortality of subjects were recorded during taking the AML leaves extract. Baseline and endline haematological parameters, such as platelets, erythrocytes, leukocytes, hemoglobin showed no significant differences, which means that the extract does not reduce the bone marrow function. Likewise, baseline and endline biochemical parameters, such as SGOT, SGPT, albumin and CRP are in normal range.[20]

Table 1: Baseline characteristic of research subjects in both groups

	Groups				Total		
Medical history	**ESFAM**		**Placebo**				p
	n	%	n	%	N	%	
Sex							0.61[t]
Male	9	69.2	8	66.7	17	68	
Female	4	30.8	4	33.3	8	32	
Age							0.83[t]
Average (year)	52.62 ± 13.77		51.5 ± 11.2		52.1± 12.3		
Stage of cancer							1[k]
Stage I	1	7.7	0	0	1	4	
Stage II	3	23.1	3	25	6	24	
Stage III	8	61.5	9	75	17	68	
Stage IV	1	7.7	0	0	1	4	
Treatment history							1[k]
During a radiotherapy and chemotherapeutic program	2	15.4	1	8.3	3	12	

	During a chemotherapeutic but not in a radiotherapy program	4	30.8	3	25	7	28	
	During a radiotherapy but not in a chemotherapeutic program	1	7.7	3	25	4	16	
	Not in a chemotherapeutic and radiotherapy program	6	46.1	5	41.7	11	44	
Body mass index								0.65[k]
	Underweight	2	15.4	3	25	5	20	
	Normal	5	38.5	7	58.3	12	48	
	Overweight	6	46.2	2	16.7	8	32	

f = Fisher test ,
k = Kolmogorov-Smirnov test
t = independent t-test

Immune response

Table 2 shows pro-inflammatory cytokines (TNF-α, IFN- γ), anti-inflammatory cytokine (IL-10) and COX-2 serum level of both groups at baseline and endline. There was no significant difference of inflammatory cytokines level (TNF-α, IFN-γ), and anti-inflammatory cytokine IL-10 between group or within groups. The COX-2 enzyme tended to decrease as compared to baseline (p<0.093), While in placebo group there was not significant (p=0.14).There was no correlation between IFN-γ and IL-10, COX-2 and IL-10 in both groups. TNF-α and IL-10 showed significant correlation in AML group after administration of the extract, but not significant in placebo group. Another significant correlation was shown by IFN-γ and IL-10, only in AML group (Table 3).

Table 2: TNF-α, IL-10, IFN-γ and COX-2 serum level in each group at baseline and endline

		AML leaves extract group†	Within group p value+	Placebo group†	Within group p value+	Between group p value‡
		n = 13 (pg/mL)		n = 12 (pg/mL)		
TNF-α	Before	11.65 (8.55 - 23.31)	0.35	12.06 (10.46 - 24.29)	0.25	0.55
	After	10.85 (10.07-15.87)		11.05 (10.46-14.56)		
IL-10	Before	9.09 ± 2.16	0.24	8.36± 1.33	0.59	0.79
	After	8.25 ± 1.22		8.05± 1.3		
IFN-γ	Before	7.26 ±7.15	0.83	7.43± 13.56	0.48	0.83
	After	6.7± 4.17		6.35 ± 4.23		
		n = 10 (ng/mL)		n = 10 (ng/mL)		
COX-2	Before	4.65 (1.87 – 16.6)	0.09	2.69 (0.68-29.59)	0.14	0.65
(ng/mL)	After	2.1 (0.66- 16.64)		2.46 (0.8 – 4.28)		

- Data are expressed as the mean ± SD for normally distributed data (IL-10 and IFN-γ) and the median (25th, 75th percentiles) for non-normally distributed data (TNF-α and COX-2).
- Paired t-test was performed for normally distributed data, Wilcoxon test for non-normally distributed data
- Independent sample t test was performed for normally distributed data, Mann-Whitney U test for non-normally distributed data
- Between group p value is statistical analysis of the difference of post-pretreatment between two groups.

Table 3: Correlation between inflammatory and anti-inflammatory responses

	Baseline		End line	
	R	p	R	p
ESFAM				
TNF-α and IL-10	0.44[s]	0.067	0.64 [s]	0.01**
IFN-γ and IL-10	-0.36[p]	0.11	0.47 [p]	0.05*

COX-2 and IL-10	0.03[s]	0.47	0.22 [s]	0.28
Placebo groups				
TNF-α and IL-10	0.00[s]	0.5	0.37[s]	0.12
IFN-γ and IL-10	0.17[s]	0.3	0.46[p]	0.07
COX-2 and IL-10	0.34[s]	0.17	-0.08[p]	0.41

P = Pearson correlation test was performed for normally distributed data
S= Spearman's Rho correlation test was performed for non-normally distributed data
* = the correlation is significant at CI = 95%

Discussion

This study is the first study on functions of ESFAM as an anti-inflammatory conducted in human. The post-resection CRC outpatient were enrolled and were administrated with 300 mg AML extract or placebo, 1 capsule a day for eight weeks.

In ESFAM group, levels of both TNF-α and IL-10 decreased, whereas in placebo group, TNF-α was decreased but IL-10 slightly increased, though not significant. The levels of IFN-γ tend to increase in both groups. In another study, the ethanolic extract of AML leaves reported to reduce levels of the pro-inflammatory cytokines TNF-α in mice, but the study conducted in only one day.8 Condition of the subjects, medical history, ages, [21,22] and BMI23,24 may contribute to variation in TNF-α serum level as pro-inflammatory cytokines.

The levels of COX-2 tended to decrease after administrating ESFAM (p=0.09) as shown in Table 2.The different therapies of the CRC outpatient subjects, chemotherapy (Capecitabine) only, radiotherapy only, both therapies and no therapy, 28% and 16%, 12 % and 44% of the subjects, respectively as shown in Table 1 may contribute to no significant decreased of COX-2 in ESFAM group.

The different therapies received by each subject may influence the level of inflammatory mediators, including COX-2. Ethanolic extract of AML leaf showed anti-inflammatory properties in animal studies, by disturbing prostaglandins biosynthesis through cyclooxygenase enzymes inhibition. Lyophilized fruit extract of AML inhibits both COX-1 and COX-2.25 In animal models, a high dose of ethanolic extract of AML leaf is needed (300 mg/kg) to demonstrate its effect in inhibiting prostaglandin in mice. Optimal dose for anti-inflammatory effect in human is not yet known. Previous research on anti-inflammatory activity in animal model used carrageenan - induced oedema model. The edema was measured every 30 minutes until four hours.

There were two phases of inflammatory mediator release during inflammatory process, the first phase took 0-2.5 hours to release histamine, bradykinin, and serotonin. After three hours, the edema was reduced. The second phase was overproduction of prostaglandins and took five hours after injection, hence, there were two peaks of curve. High dose of AML extract worked in both of two phases, and the result is comparable with positive control (NSAIDs), it was concluded that the anti-inflammatory effect of ethanol fraction of AML leaves extract might be working on prostaglandin synthesis pathway. There haven't been any AML studies by directly measure the COX-2 levels and prostaglandin.[15]

A significant positive correlation between TNF-α and IL-10 ($p = 0.01$, $R = 0.64$) was found. Likewise, a significant positive correlation between IFN-γ and IL-10 in AML group was found ($p = 0.05$, $R = 0.47$). These facts suggest that inflammatory compensation process runs.

In normal individual, when an inflammation process occurred, and the pro-inflammatory cytokine level increased (including TNF-α), there should be an immune mechanism to restore the inflammatory conditions back to a balance of inflammatory and anti-inflammatory immune response. The equilibrium levels of inflammatory cytokines and anti-inflammatory will protect the

individual from various pathological mechanisms caused by the inflammatory process including colitis-associated colorectal cancer.Chronic inflammation occurs when there is imbalance between pro-inflammatory and anti-inflammatory response, the balance between Th17 cells and Treg cells played important role. Naïve T cells differentiate into effector cells based on the micro environment cytokines.

TGF-ß alone can trigger the differentiation of naive T cells into Treg cells, but with the addition of pro-inflammatory cytokines, especially IL-6, may change the naïve T cell differentiation into Th17 cells.[26-28] Existing Treg cells can also be further differentiate into Th17 cells in the presence of TGF-ß plus pro-inflammatory cytokines IL-1ß, IL-21 and IL-[23.28,29] More naïve Tc ells differentiate into Th17 cells will drive the balance toward pro-inflammatory response.

Interleukin-6 is a cytokine secreted by monocytes, macrophages and colon cancer cells. High levels of IL-6 serum associated with tumor progression, metastasis and poor prognosis.30 AML leaf extract was reported for its ability in inhibiting the pro-inflammatory cytokine IL-6 and TNF-α.[31] Besides IL-6 inhibition, flavanes contained in AML leaves could stimulate IL-2 secretion.[17,32] IL-2 also promote the generation of Treg cells through the transcription factor STAT5. Decreasing of IL-6 and increasing of IL-2 may stimulate Treg cells generation and increase of IL-10 production to compensate the pro-inflammatory response.

IL-10 and IFN-γ also indicated a positive significant correlation (p = 0.05 and R = 0.47). IFN-γ was known as one of cytokine which acts as TNF-α inducer.[33] Referring to another result, it could be due to ability of IFN-γin increasing TNF-α, followed by an increase of IL-10 by TNF- α.

Conclusion

Taken together, the activation of anti-inflammatory pathways in response to inflammatory condition leading to normal homeostasis in out-patient subjects with colorectal cancer administered with AML ethanolic fraction.

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Conflict of Interests

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

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