ORIGINAL ARTICLE

Chemopreventive Effect of Carica Pubescens Leaf Extract on Neutrophil-lymphocyte Ratio, Erythrocyte Count, and Colon Histopathological Appearance of Dimethylhydrazine-induced Colon Cancer Rats

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ABSTRACT

Introduction: Colorectal cancer is a disease that occurs from three major pathways: sporadic, hereditary, and inflammatory. Carica pubescens (CP) have potential chemopreventive effects. This study wants to determine the effect of CP leaf extract on the neutrophil-lymphocyte ratio (NLR), erythrocyte count, and colon histopathological appearance of rats induced by dimethylhydrazine (DMH). **Methods:** The samples were Sprague Dawley rats (n=35, male, 5-7 weeks aged, 150-200 grams). NC was the normal control group. The DMH group was induced by 20 mg/kg BW of DMH (subcutaneously) once a week for 15 weeks. CP75, CP150, and CP300 were induced by DMH and given CP leaf extract orally at doses of 75, 150, and 300 mg/kg BW, once daily for 15 weeks. The neutrophil, lymphocyte, and erythrocyte count were measured using hematology analyzer. The histopathological appearance shows the percentage of total aberrant crypt foci, and the degrees of inflammation cells. **Results:** The NLR of CP75 (0,23±0,042), CP150 (0,26±0,018), and CP300 (0,21±0,039) was significantly lower than DMH (0,64±0,239), p<0,001. The erythrocyte count of CP150 (8,89±1,082 x106 cell/µL; p=0,034) and CP300 (9,24±0,396 $x10^6$ cell/µL; p=0,006) was significantly higher than DMH (7,87±0,641 $x10^6$ cell/µL). The percentage of total aberrant crypt foci of CP75 (15,71±5,345%,p=0,006), CP150 (18,57±6,901%,p=0,014), and CP300 (5,71±5,345%,p=0,001) was significantly lower than DMH (59,29±32,714%). **Conclusion:** Carica pubescens leaf extract may reduce the neutrophil-lymphocyte ratio, percentage of total aberrant crypt foci and inflammation degree in colon histopathological appearance, otherwise increase the number of erythrocyte in DMH-induced colon cancer rats.

Keywords: Carica pubescens, Colon cancer, Dimethylhydrazine, NLR

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INTRODUCTION

"Colorectal cancer (CRC) is the fourth leading cause of cancer death and the third most commonly diagnosed cancer worldwide by 2018" (1–3). According to the Global Burden of Cancer (Globocan) 2018, there were 1,8 million new cases (10,2% of the total) and 881.000 deaths (9,2% of the total) caused by colorectal cancer in 2018 and will increase to more than 2.2 million new cases and 1.1 million deaths by 2030 (3,4). "In the US, there will be an estimated 104,610 new cases of colon cancer and 43,340 cases of rectal cancer diagnosed in 2020" (2 p.3).

Colorectal cancer is a heterogeneous disease that occurs at least from three major pathways: sporadic, hereditary, and inflammatory. All three causes different genetic changes despite having some mutation similarities. The inflammatory process plays a role in the mechanism of cancer and may come from some factors, including environmental carcinogens, infection,

cellular senescence, and obesity. Increased production of leukocytes and proinflammatory cytokines are some hallmarks of systemic inflammation. (5–8). Several studies have shown the correlation between inflammation process and the risk of colorectal cancer (5–9).

Neutrophil-lymphocyte ratio (NLR) is one of the parameter to measure a systemic inflammation and investigate the chronic condition (10–12). A study from Imtiaz F et.al stated that systemic inflammation measured by NLR has significant association with prevalent chronic conditions in hypertension and diabetic patients (13). Another study from Stojkovic Lalosevic et.al stated that NLR is potential biomarker in diagnostic and early recognition of different stages in colorectal cancer (10).

These malignancies also have a direct effect on anemia. Anemia of inflammation (AI), also known as anemia of chronic disease (ACD) correlates with increased levels of cytokines, which are usually observed in infections, inflammatory diseases, and cancer (14–16). Erythrocyte count is one of parameter to identify anemia of chronic disease. Kolte et.al proved that there were a lower erythrocyte count, an increased level of total leukocyte count, neutrophil, lymphocyte, and eosinophil count in the 100 patients with chronic periodontitis, compared to the healthy control group (17). Weiss et.al stated that chronic and sytemic inflammation can shortened erythrocyte half-life, suppressed erythropoietin response, and inhibit erythroid cell differentiation (16).

We also do a colon histopathological examination to confirm the process of colon cancer progression. The histopathological appearance shows the percentage of total aberrant crypt foci (ACF) and the degrees of inflammation cells. ACF is the pre-lesion in colon cancer development and can be seen microscopically in the colon mucosa (18).

One of the common carcinogenic agents used in colorectal cancer research is dimethylhydrazine (DMH). DMH is often used to induce colorectal cancer in rodents through the DNA methylation process in the compartment proliferation of crypts, which resulted in the loss of many colon cells to apoptosis and increased colonic epithelial cell mutations (19,20).

The need to find alternative anticancer therapy and chemopreventive agent is triggered by the fact that most available treatment modalities such as chemotherapy, radiotherapy, and surgery can give severe side effects, e.g. hair loss, immunosuppressants, diarrhea, and bleeding. In addition, an advanced stage of colorectal cancer patients have a low survival rate and a poor prognosis. Therefore, efforts are now being made for the prevention of colorectal cancer. Chemopreventive agents are becoming important in colorectal cancer treatment. Early intervention in colorectal premalignant lesions with natural products can be a promising and cost-effective approach, especially for the prevention of colorectal cancer in a sizeable population. Herbal medicine or natural product is expected to be an alternative to effective adjuvant therapy and chemoprevention with minimal side effects (21–23).

Carica pubescens, is a type of fruit plant that was cultivated and adapted to highland environments. In Indonesia, this plant is found in Cangar, Bromo, and Dieng highlands, Central Java (24). This species is a member of the *Caricaceae* family, so it has the same genus group with *Carica papaya* and has similarities in morphology. Unlike the papaya, this plant grows in places with an altitude of 1,400-2400 meters above sea level (asl) and low temperature. Qualitative test results through phytochemical screening of *Carica pubescens* which grew in the Cangar, Bromo, and Dieng highlands areas showed that the samples were positive for flavonoids, polyphenols, tannins, and triterpenoids (24,25). *Carica papaya* leaf extract which contains active components such as flavonoids, can inhibit tumor cell growth and stimulate anti-tumor activity (26). This research wants to determine the chemopreventive effect of CP leaf extract on the neutrophil-lymphocyte ratio (NLR), erythrocyte count, and colon histopathological appearance of rats induced by DMH.

MATERIALS AND METHODS

Samples and Experimental Design

This experiment used 35 Sprague dawley rats, aged 5-7 weeks, weighed 150-200 grams, and obtained from Gajah Mada University, Yogyakarta, Indonesia. After simple random allocation, rats were grouped into five with seven rats each. NC was the control group. The DMH group was induced by 20 mg/kg BW of DMH (subcutaneously) once a week for 15 weeks. CP-75, CP-150, and CP-300 group was induced by DMH and administered by 75, 150, and 300 mg/kg BW CP leaf extract (orally), once daily for 15 weeks.

Carica pubescens Leaf Extraction

Carica pubescens leaves were harvested from Dieng, Banjarnegara, Central Java, Indonesia, then dried and ground into a powder using mortar and pestle. Powder samples were extracted by cold maceration technique using ethanol. The maceration product was filtered and evaporated in a regulated water bath (maintained at 50° C) to produce a dark green semi-solid extract. The extract was stored in a refrigerator at 4° C and prepared for oral administration.

Neutrophil, Lymphocyte, and Erythrocyte Count

Blood samples from retroorbital plexus were taken at the end of the experiment day, and put in EDTA tube. The neutrophil, lymphocyte, and erythrocyte count were measured using Sysmex KX-21 hematology analyzer. The neutrophil-lymphocyte ratio (NLR) was obtained

from the calculation of neutrophil absolute count divided by lymphocyte absolute count.

Colon Histopathological Examination

At the end of experiment, the rats were euthanized by cervical dislocation. Immediately after the euthanasia, the colons were removed, elongated, and washed with saline, then fixed with 10% formalin buffer and stained with Haematoxylin and Eosin (H&E) staining. The sample was viewed using light microscope with 100x and 400x magnifications. The percentage of aberrant crypt foci is obtained by calculating the number of aberrant crypts compared to the total number of crypts. The degrees of inflammation cells were scored as follows: score 1: there is no distribution of lymphocyte and neutrophil cells; score 2: mild distribution of lymphocyte and neutrophil cells; score 3: moderate distribution of lymphocyte and neutrophil cells; score 4: severe distribution of lymphocyte and neutrophil cells. Reading is carried out by two anatomical pathologists.

Statistical Analysis

Analysis of data normality using the Shapiro-Wilk test. The neutrophil-lymphocyte ratio and erythrocyte count were analyzed using one-way ANOVA test. After obtaining a significant result, the analysis was continued with post hoc LSD. The percentage of total aberrant crypt foci and the inflammation cells degree in colon histopathological appearance were analyzed using the Kruskal-Wallis test. After obtaining a significant result, the analysis was continued with Mann-Whitney test. The analysis was performed with SPSS version 25.0 and graphed using GraphPad Prism software.

Ethical Approval

Ethical approval was obtained from the Ethics Committee for Medical Research of Faculty of Medicine Diponegoro University/ Dr. Kariadi Semarang (EC No. 100/EC/H/FK-RSDK/VIII/2018).

RESULTS

Carica Pubescens Leaf Extract and Animal Weight

From the qualitative and quantitative analysis of CP leaf extract using spectrophotometry, the extract shows the presence of flavonoid (16,3%), tannin (37,65%), saponin (10,4%), alkaloid (4,23%), and terpenoid. Before starting the experiment, we calculate the mean of body weight from all of the groups. Mean of body weight in NC group was 177,43±12,246 grams, DMH group was 175,29±6,550 grams, while in the CP75, CP150, and CP300 was 178,14±6,440; 171,29±11,954; and 176,29±11,586 grams. It qualified the homogeneity (Lavene's test *p=0,239*) and the normality data (Shapiro Wilk *p=0,205*).

Neutrophil-Lymphocyte Ratio (NLR) and Erythrocyte Count

The result and comparative analysis of NLR between groups can be seen in Table I and Figure 1-A. This study found that the highest neutrophil-lymphocyte ratio (NLR) was in DMH group (0,64±0,239), while the lowest was in the NC group (0,20±0,087). Among the *Carica pubcencens* group, the lowest NLR was in the CP300 group (0,21±0,039). The NLR of CP75 (0,23±0,042), CP150 (0,26±0,018), and CP300 (0,21±0,039) was significantly lower than DMH group (0,64±0,239), p<0,001.

Table I. Neutrophil-Lymphocyte Ratio (NLR) in All Groups

Groups	n	NLR	*p*
NC	7	0,20 ± 0,087	
DMH	7	0,64 ± 0,239	
CP75	7	0,23 ± 0,042	p < 0,001*
CP150	7	0,26 ± 0,018	
CP300	7	0,21 ± 0,039	

The table shows Means ± Standard Deviation
*One Way ANOVA (p<0.05 was considered significant)
NC=Normal Control group, DMH=Dimethylhydrazine group
CP75, CP150, CP300= Carica pubescens group with 75, 150, and 300 mg/kg BW doses

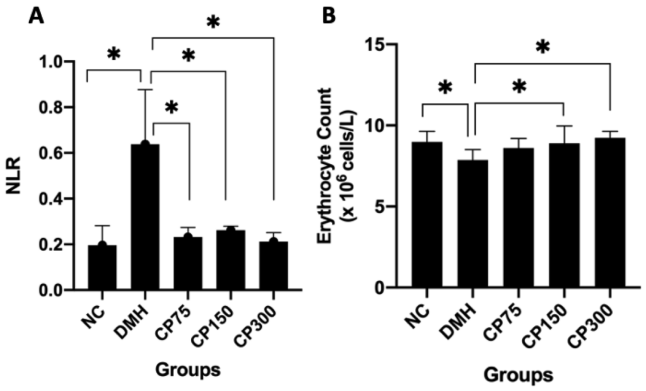

Figure 1 : Comparative Analysis of Neutrophil-Lymphocyte Ratio (NLR) and Erythrocyte Count Between Groups
(A). The comparative analysis of NLR between groups. (B). The comparative analysis of erythrocyte count (x10^6 cell/µL) between groups. The comparative analysis between groups was assessed using post hoc LSD. Data are given as means ± SD (n = 7 per group).

Table II and Figure 1-B describe the erythrocyte count and the comparative analysis between groups. The lowest value was seen in the DMH group (7,87±0,641 x10^6 cell/µL), while the highest one was in the CP300 group (9,24±0,396 x10^6 cell/µL). The erythrocyte count of CP150 (8,89±1,082 x10^6 cell/µL; p=0,034) and CP300 (9,24±0,396 x10^6 cell/µL; p=0,006) was significantly higher than DMH (7,87±0,641 x10^6 cell/µL). There was no difference between DMH and CP75 group.

Table II : Erythrocyte Count in All Groups

Groups	n	Erythrocyte Count (x 10^6 cells/µL)	*p*
NC	7	8,98 ± 0,653	
DMH	7	7,87 ± 0,641	
CP75	7	8,60 ± 0,592	*p = 0,058**
CP150	7	8,89 ± 1,082	
CP300	7	9,24 ± 0,396	

The table shows Means ± Standard Deviatio
*One Way ANOVA (p<0.05 was considered significant)
NC=Normal Control group, DMH=Dimethylhydrazine group
CP75, CP150, CP300= Carica pubescens group with 75, 150, and 300 mg/kg BW doses

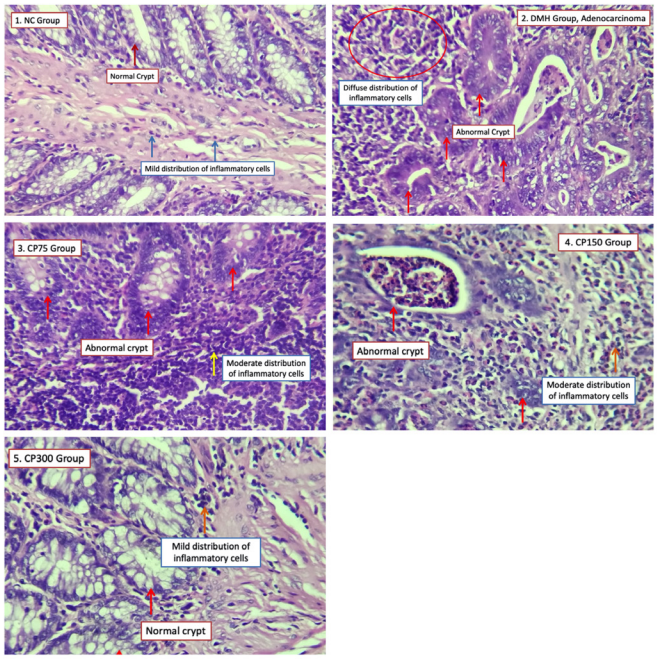

Figure 2 : Colon Histopathological Appearance in All Groups
The colon histopathological appearance was shown from the percentage of total ACF and the inflammation cells degree. The sample was given Haematoxylin and Eosin (H&E) staining and was viewed using light microscope with 400x magnifications.

Colon Histopathological Examination

The colon histopathological appearance seen from the percentage of total aberrant crypt foci (ACF) and the degrees of inflammation cells were analyzed by two anatomical pathologists. The microscopic result shows in Figure 2. There were abnormal crypts and diffuse inflammatory cells in the DMH group. In the CP75 and CP150 group, we can see abnormal crypt and moderate inflammation, while in the CP300 group we only see mild inflammation.

Table III and Figure 3-A show the percentage of total ACF and comparative analysis between groups. From Table III, it can be seen that the lowest percentage of total ACF was in the CP300 group (5,71±5,345 %), while the highest was in the DMH group (59,29±32,714 %). The comparative analysis in Figure 3-A shows that the total ACF percentage in the DMH group was significantly higher than CP75 (p=0,006), CP150 (p=0,014), and CP300 group (p=0,001).

The comparative of inflammation degree between groups can be seen in Figure 3-B. The mild, moderate, and diffuse inflammation was presented in the DMH group, while in the CP group only found mild and moderate inflammation.

Table III : The Percentage of Total Aberrant Crypt Foci (ACF) in All Groups

Groups	The percentage of total aberrant crypt foci		*p*
	Means ± SD	Median (min-max)	
NC	5,71 ± 5,345	10 (0 - 10)	
DMH	59,29 ± 32,714	50 (20 - 95)	*p < 0,001**
CP75	15,71 ± 5,345	20 (10 - 20)	
CP150	18,57 ± 6,901	20 (10 - 30)	
CP300	5,71 ± 5,345	10 (0 - 10)	

*Kruskal Wallis (p<0.05 was considered significant)
NC=Normal Control group, DMH=Dimethylhydrazine group
CP75, CP150, CP300= Carica pubescens group with 75, 150, and 300 mg/kg BW doses

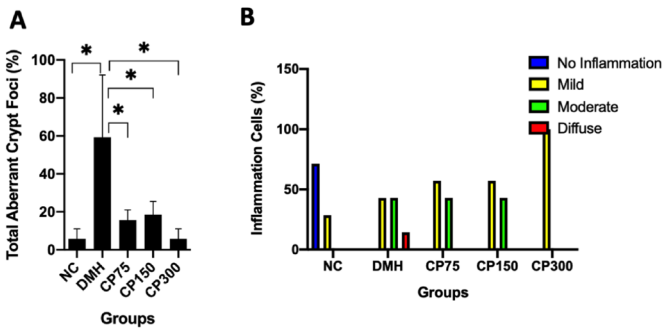

Figure 3 : Comparative Analysis of Colon Histopathological Appearance Between Groups
(A). The comparative analysis of total aberrant crypt foci (%) between groups. The comparative analysis between groups was assessed using Mann Whitney test. Data are given as means ± SD (n = 7 per group). (B). The comparative analysis of inflammation cells degree between groups. Data are given as percentage of inflammatory cells in each degree in all groups.

Correlation Between Neutrophil-Lymphocyte Ratio and Colon Histopathological Appearance

Figure 4 shows the correlation diagrams of NLR and the percentage of total ACF. From Figure 4 we can see a significant correlation between NLR and the percentage of total ACF (*p*= 0,005; *r*=0,465). A positive correlation indicates that higher NLR is associated with a higher percentage of total ACF in colon histopathological appearance.

DISCUSSION

The process of colorectal cancer from normal colon epithelium requires inflammatory factors and a series of genetic processes to activate and form a tumorigenic environment (5,7,8,27–29). Chronic inflammation,

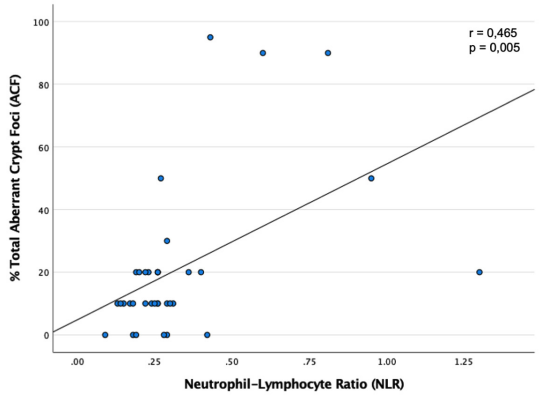

Figure 4 : Correlation Between Neutrophil-Lymphocyte Ratio (NLR) and Percentage of Total Aberrant Crypt Foci (ACF)
The correlation between NLR and % of total ACF was assessed using Spearman correlation test (n = 7 per group).

one of an important factor in colorectal cancer (CRC) development, triggers the production of pro-inflammatory cytokines and oncogenes, resulted in an increased number of white blood cells, lymphocytes, neutrophils, and the progression of tumorigenesis. CRC is caused by mutations of tumor suppressor genes and the accumulation of oncogenes, leads to aberrant activation of β-catenin signaling. Mutations in β-catenin, adenomatous polyposis coli (APC), or other components mediate the transition of single pre-neoplastic cells to aberrant crypt foci (ACF) and later to adenoma and colorectal carcinoma (30). ACF is the pre-lesion in colon cancer development and can be seen microscopically in the colon mucosa (18). Colitis-associated cancer (CAC) that come from chronic inflammation process has been studied to understand the mechanism of transition from the inflammatory process to malignancy (28,30).

In this study, there is an increasing number of neutrophil and lymphocyte count in the blood of the DMH group. We can also see an increase in total ACF and inflammation cells in the colon histopathological appearance of DMH group. These results were the same as the previous study stated that DMH is a potential agent for colorectal cancer induction (18,31–33). DMH can induce oxidative stress production, inflammation process, and tumor progression in the colon of the animal model (31). DMH is often used to induce colorectal cancer in rodents through the DNA methylation process in the compartment proliferation of crypts, which resulted in the loss of many colon cells to apoptosis and increased colonic epithelial cell mutations (19,20).

Chemoprevention by natural products can be a potential approach to prevent the development of cancer. The result of this study proved that *Carica pubescens* (CP) leaf extract with a multilevel dose of 75, 150, and 300 mg/kg BW can reduce the neutrophil-lymphocyte ratio, and significantly lower the inflammation degree and percentage of total aberrant crypt foci in colon histopathological appearance, compared with the DMH group. This result proved the anti-inflammatory and chemopreventive effect of *Carica pubescens* in rats induced colon cancer.

Carica pubescens has the same genus group with *Carica papaya*. From the spectrophotometry analysis, CP leaves extract contains flavonoid, tannin, saponin, alkaloid, and terpenoid. Previous study stated that *Carica papaya* leaf extract can inhibit tumor cell growth and stimulate anti-tumor activity (26). The flavonoid-rich contain of papaya seed extract can inhibit the expression of proinflammatory cytokines such as IFNγ, TNFα, IL-6, and NF-kB (34,35). Another study from Salim et al showed an anti-inflammatory effect of papaya leaf extract (5 mg/ml) by inhibiting the production of pro-inflammatory TNFα, IL-1α, IL-1β, IL-6, and IL-8 (35,36).

This study also found that *Carica pubescens* (CP) leaf extract with dose of 150 and 300 mg/kg BW can increase the number of erythrocytes compared with the DMH group. This malignancy has a direct effect on anemia. Anemia of chronic disease correlates with increased levels of cytokines, which are usually observed in infections, inflammatory diseases, and cancer (14,15). The result of this study is consistent with the study conducted by Sule OJ et al which shown that *Carica papaya* L, may increase erythropoietic properties on rats pretreated groups through increased levels of hemoglobin (Hb) and red blood cells (RBC) (37). Another study from Dharmarathna et al also proved that fresh *Carica papaya* leaves can significantly increase RBC count and platelet count (38).

Further research is needed to investigate the chemopreventive effect of *Carica pubescens* in colon cancer and the underlying mechanism. More parameters are needed to assess, such as proinflammatory cytokines (IFNγ, TNFα, IL-2, IL-6), or other inflammatory networks related to colon cancer.

CONCLUSION

Carica pubescens leaf extract may reduce the neutrophil-lymphocyte ratio, percentage of total ACF and inflammation degree in colon histopathological appearance, otherwise increase the number of erythrocyte in DMH-induced colon cancer rats.

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