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Phyllanthus niruri Linn increase infiltrating lymphocyte and apoptosis of rectosigmoid cancer patients: A Phase II Clinical Trial

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ABSTRACT

Background: Phyllanthus niruri Linn (PNL) extract has potential cytotoxic effects on colorectal cancer in both in vitro and in vivo studies, but this has never been reported in human subjects. The purpose of this study is to elaborate whether PNL extract also has cytotoxic effects on human colorectal cancer.

Method: A one-group pre- and post-test analysis was conducted on 15 patients with non-obstructive operable rectosigmoid cancer without neo-adjuvant chemotherapy or chemoradiation. Before commencing treatment using PNL extract (100 mg Stimuno), all patients underwent rectoscopy biopsy. Stimuno treatment was given for at least 14 days and later patients underwent rectal resection. Specimens from biopsy and rectosigmoid resection were managed by the Department of Anatomical Pathology, Faculty of Medicine, Gajahmada University, Yogyakarta, for evaluating the diagnosis, infiltrating lymphocyte, apoptosis index, perforin, and granzyme-B and caspase-3 expression. A paired *t*-test was done to evaluate the significant increase in measured variables, and a linear regression analysis was also done to evaluate the role of individual variables.

Result: Infiltrating lymphocyte; expression of perforin, granzyme-B, and caspase-3; and apoptosis index significantly ($p = 0.000$) improved after the treatment using PNL extract. After conducting the linear regression analysis, we found that the only variable that significantly correlated with apoptosis was infiltrating lymphocyte.

Conclusion: Use of PNL extract increased the apoptosis index of colorectal cancer through an increase in infiltrating lymphocyte.

Keyword: Phyllanthus niruri Linn, rectal cancer, infiltrating lymphocyte, apoptosis

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INTRODUCTION

Neo-adjuvant or adjuvant treatment of colorectal cancer has reduced the recurrence rate and improved the 5-year survival rate. A study of 119,363 colon cancer cases between January 1991 and December 2000 showed that according to American Joint Committee on Cancer (AJCC) TNM System Sixth Edition stages, the 5-year-stage-specific survival rate was 93.2% for stage I, 84.7% for stage IIa, 72.2% for stage IIb, 83.4% for stage IIIa, 64.1% for stage IIIb, 44.3% for stage IIIc, and 8.1% for stage IV. The significantly better result of stage IIIa compared to that of Stage IIb could be attributed to the use of chemotherapy. National Institutes of Health (NIH) 1990 Consensus Conference guidelines for colorectal cancer recommend adjuvant chemotherapy for stage III colon cancer but not for stage II patients.[1] Review done by El Zouhairi et al. 2011[2] showed that over the past decade, the survival rate of metastatic colorectal cancer patients improved dramatically after using chemotherapy. In the mid-1990s, the median overall survival rate of those treated with 5-FU-based regimen was about 12 months; the combination of 5-FU with irinotecan or oxaliplatin increased the OS to approximately 18%, and after addition with targeted therapy, the OS approached 30% in some studies. Although there was a lot of improvement in survival, the results of stage IIIb, IIIc, and IV were still disappointing.

Thus, these disappointing results that came about even after the treatment using modern chemo for targeted therapy of metastatic colorectal cancer stimulated research into finding substances that may solve this problem. Immunomodulator seems to be the other alternative to improve survival and reduce the recurrence of colorectal cancer. Phyllanthus niruri Linn (PNL) is one of the potent immunomodulator; its efficacy has already been proven in relation to its capacity to inhibit the development of experimental colorectal cancer of Sprague-Dawley male rat. PNL extract significantly decreased AgNORs, the cancer cells' proliferative marker, and macroscopic tumor growth.[3] In vitro study showed that spray-dried extracts of Phyllanthus niruri (SDEPN) has a significantly better cytotoxic effect on Hepatocellular cell cancer line (Hep G2) but not on colorectal cancer cell line

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(HT29) compared to control. However, pretreatment with SDEPN followed by cisplatin had a synergistic effect and showed a significantly much better cytotoxic effect on HT 29.[4] PNL extract has already been used widely as traditional medicine in Indonesia. In experimental animal model PNL is used for the treatment of kidney or liver, even in extremely high doses at >5000 mg/kg. b.w.[5]

The purpose of this study is to evaluate whether PNL extract can also influence the growth of colorectal cancer patients through perforin–granzyme pathway, by measuring infiltrating lymphocyte, perforin, granzyme-B, caspase-3, and apoptosis index before and after the PNL extract treatment.

METHOD

The study sample included patients with resectable non-obstructive rectal cancer but who did not undergo neo-adjuvant chemotherapy or chemo-radiation; other selection criteria included the following: age above 40 years, a BMI score that is more than 20, and hemoglobin content that is more than 10 gr%. Patients were explained about the study, and informed consent was obtained from them; eventually, a total of 15 rectal cancer patients were enrolled in the present study which was conducted in Dr. Kariadi Hospital, Semarang Indonesia, between May and July 2016. The study has already been approved by the Ethical Clearance Committee of Dr. Kariadi Hospital, Faculty of Medicine, Diponegoro University. The study was based on pre-test and post-test design. All patients underwent rectoscopy and biopsy and were managed as outpatients. The biopsy specimen was fixed with formalin buffer within 18–24 hours and further processed with paraffin block. The paraffin block was sent to Anatomical Pathology Laboratory of Faculty of Medicine, Gajahmada University, for further analysis regarding the microscopic diagnosis and immune histochemical staining for detecting perforin, granzyme-B, caspase-3 expression, and degree of apoptosis of cancer cells. The next day after rectoscopy the patient was treated with PNL extract (R/Stimuno) 100 mg/day for a minimum 14 days. Stimuno contains a single composition formula of Phyllanthus niruri plant extracts taken from roots, leaves, stems, and fruit and formulated as capsules. Stimuno is being used since 1999 ; we obtained a certificate of phytopharmaca by POM RI on March 22, 2005. After Stimuno treatment for 14 days or more, patients were admitted to Dr. Kariadi Hospital and underwent rectal resection, and specimens were treated as biopsy specimen in the same Anatomical Pathology Laboratory. Infiltrating lymphocyte was measured on Hematoxylin Eosin staining specimen under 400x power field. The apoptosis index was measured using the terminal deoxynucleotide transferase dUTP nick-end labeling method. Perforin, granzyme-B, and caspase-3 expressions were measured on immune histochemical staining specimen under 400x power field. All were measured by two independent anatomical pathology experts using the same criteria and clinical agreement was more than 95%. As the numerical data showed normal distribution, paired *t*-test was used to compare variables used in the study before and after treatment. Linear regression analysis of the increasing value of variables was done to identify which variable significantly predicted the increased apoptosis.

RESULT

All the 15 rectal or sigmoid cancer patients completed the study. The mean length of treatment with stimuno was 17 days (14–22 days). Patients gender classification was as follows: 9 males and 6 females; mean of age of the patients was 53.66 ± 14.52 years. Mean serum urea level pre-treatment was 27.8 ± 6.48 mg/dl, range 17–38, and post-treatment the same was 29.9 ±7.78 mg/dl, range 19–42 (p = 0.000). Mean serum creatinine pre-treatment was 0.89 ± 0.22 mg/dl, range 0.56–1.20, and post-treatment it was 0.85 ± 0.18 mg/dl, range 0.56–1.35 (p = 0.000). Serum alanine aminotransferase (ALT) pre-treatment was 28.3 ±7.71 unit/l, range 17–36, and post-treatment it was 26.8 ±6.38 unit/l, range 17–40 (p = 0.000).

Infiltrating Lymphocyte, granzyme-B, perforin, caspase-3 expression, and apoptosis index significantly increased after treatment with PNL extract (Table 1).

To know the relationship among variables, since it was a pre- and post-test design, a correlation matrix of increasing value of variables (the

Table 1 Variables before and after PNL extract treatment *paired *t*-test

No	Variables		Mean (SD)	p*
1	Lymphocyte	pre	45.4 (4.8)	0.000
		post	179.8 (38.8)	
2	Granzyme-B	pre	25.4 (4.8)	0.000
		post	65.7 (7.9)	
3	Perforin	pre	37.6 (20.7)	0.000
		post	42.0 (20.9)	
4	Caspase-3	pre	37.4 (13.2)	0.000
		post	61.0 (18.3)	
5	Apoptosis	pre	16.9 (5.0)	0.000
		post	87.2 (20.3)	

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Table 2 Correlation matrix among increasing variables. r: Pearson Correlation, p (significant level two-tail), *Infiltrating

Variables		*Lymphocyte	Granzyme-B	Perforin	Caspase-3	Apoptosis
*Lymphocyte	r	1	0.15	-0.03	0.46	0.71
	p	0.589	0.912	0.080	0.003	
Granzyme-B	r	0.15	1	0.25	-0.12	0.18
	p	0.589	0.363	0.667	0.502	
Perforin	r	-0.03	0.25	1	-0.08	0.14
	p	0.912	0.363	0.753	0.613	
Caspase-3	r	0.46	-0.12	-0.08	1	0.31
	p	0.08	0.667	0.753	0.255	
Apoptosis	r	0.71	0.18	0.14	0.31	1
	p	0.003	0.502	0.613	0.255	

Table 3 Linear regression with dependent variables increasing apoptosis, while increasing infiltrating lymphocyte, granzyme-B, perforin, and caspase-3 expression were independent variables

	Unstandardized Coefficients	Standardized Coefficients	*t*	
Model Significance	**B**	***SE***	**Beta**	
Constant	17.753	14.845	1.196	0.253
Lymphocyte diff.	0.391	0.106 0.715	3.690	0.003

different between post- and pre-test values) was done. It showed that the one variable that strongly correlated with apoptosis was lymphocyte (r = 0.715, p = 0.003). A nearly significant correlation was shown between infiltrating lymphocyte and caspase-3 (r = 0.46, p = 0.080; Table 2). On linear regression, it was more clear that apoptosis was strongly correlated with only lymphocyte (Table 3).

DISCUSSION

This study was phase II clinical trial, and the study purpose was to know whether the extract of PNL, which was already proven to be effective in arresting the growth of experimental Windstar colorectal cancer,[3] also has a positive effect on arresting human colorectal cancer growth. The research regarding PNL's capacity to arrest the growth of human colorectal cancer cells has never been reported. Since PNL extract has already been used widely in the Indonesian community as a traditional medicine and proven save, the Phase I clinical trial was not done. This argument is supported by the result of this study. Although serum of urea, creatinine, and ALT changed significantly, the levels of both pre- and post-PNL treatment were still within normal range. It means that PNL is safe for kidney and liver, which is in accordance with the previous research[5]. The results of the study showed that PNL extract significantly increased the infiltrating lymphocyte, granzyme-B, perforin, caspase-3 expression, and cancer cell apoptosis. These results are supported by several studies that used PNL in arresting the development of various cancer cells.

Past studies using PNL on hepatic carcinoma or prostatic cancer cell line reported results that agree with the findings of this study. Cell cytotoxicity assays of Phyllanthus niruri extract showed that the active component is corilagin, which has a broad-spectrum antitumor activity to arrest hepatic carcinoma cell line, better antitumor potential, and lower toxicity in normal cells.[6] The other study on hepatic carcinoma cell line showed that SDEPN reduces the viable liver cancer cell and increases apoptosis.[4] Both aqueous and methanolic extracts of PNL significantly increase pro-apoptotic protein (Bax) and decrease anti-apoptotic protein (Bcl-2) of the prostate cancer (PC-3) cell line. NfKB also decreases significantly with both aqueous and methanolic extract of PNL. Bax protein could induce cytochrome □ release from mitochondria, which can then induce proteolytic activation of procaspase-9. This in turn activates caspase-3 and -7 and finally leads to induction of apoptosis in PC-3 cells.[7]

Studies that evaluated the effect of PNL on colorectal cancer have used only animal experimental model and have been conducted only in vitro. Study on Winstar rat showed that use of 1,2 DMH (Dimethylhydrazine) led to the development of colorectal cancer and that PNL extracts reduced the AgNOR as a marker of cell proliferative and also

reduced the size of the tumor.[3] In combination with cisplatin, SDEPN had a synergistic effect and showed significantly much better cytotoxic effect on colorectal cancer cell line (HT 29) compared to cisplatin or SDEPN alone.[8] The other study for colorectal cancer was using Phyllanthus watsonii. In vitro study on human gynecologic and colon cancer cells line showed that P watsonii arrested cell cycle at different growth phases and increased the Caspase-3 activity as a single agent or in combination with doxorubicin. It can be concluded that the sub-fraction of P. watsonii strongly inhibits the growth and induces apoptosis of human gynecologic and colorectal cancer.[9] Downregulation of anti-apoptotic protein (BCL-2) is comparable between P. watsonii and P niruri on the study done in vitro with lung cancer cell line.[10]

There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. There is an additional pathway that involves T-cell-mediated cytotoxicity and perforin-granzyme-dependent killing of the cell.[11] In this study, the role of PNL extract as an apoptotic agent was measured through perforin-granzyme-dependent killing pathway. The strong correlation was only shown between infiltrating lymphocyte and apoptosis index. The relationship between infiltrating lymphocyte and caspase-3 was almost significant; meanwhile, increased expression of perforin and granzyme-B was not significantly correlated to the apoptosis and also to infiltrating lymphocyte. Granzymes that play an important role in the apoptosis mechanism are granzyme A and granzyme B. The extrinsic, intrinsic, and granzyme B pathways converge on the same execution pathway that is the cleavage of caspase-3, as executor of apoptosis. Caspase-6 and -7 are the other executors of apoptosis. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single-stranded DNA damage. Granzyme A cannot activate caspase but can kill cancer cells by the direct cleavage of nuclear proteins, thereby facilitating the formation and accumulation of single-stranded DNA breaks.[11] In this study, granzyme A was not measured, and measuring infiltrating lymphocyte as a whole means it consists of many types of subset lymphocyte; meanwhile, lymphocytes that are capable of initiating apoptosis are only cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. To kill the cell target needs direct contact. Therefore, only a part of infiltrating lymphocyte expresses perforin and granzyme.[12] The above explanation makes it evident that an increase in PNL content arrests the increase in infiltrating lymphocyte and apoptosis of rectosigmoid cancer partly through perforin and granzyme-B pathways and granzyme-A, but extrinsic pathway still needs to be studied further. Further studies regarding the role of PNL needs to be more specific to lymphocyte, CTLs, and NK cells should be carried out.

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor–mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily.[11] Study done by Reissfelder et al. (2015)[13] showed that upregulation of TNF-α expression in tumor-infiltrating lymphocytes (TILs) strongly correlated with an increase in the total amount of intratumoral TNF-α, which is indicative of tumor-specific CTL activity. Increased TNF-α concentration was an independent prognostic factor. It suggests that intratumoral TNF-α might be a more suitable factor for prognosis prediction in patients with CRC than the quantification of effector TC infiltrates.

The relationship between infiltrating lymphocyte and the survival of colorectal cancer has already been studied. The low tumor-infiltrating lymphocyte (TIL) group was significantly correlated with a poorly differentiated status and perineural invasion. During the median 54-month follow-up period, the low-TIL group had a significantly lower five-year overall survival and disease-free survival rates than the high-TIL group of patients with stage III colorectal cancer ($p = 0.005$ and $p = 0.03$, respectively).[14] Meta-analysis of 12 publications regarding tumor inflammatory infiltrate showed that high generalized tumor inflammatory infiltrate was associated with good overall survival (HR: 0.59, 95% CI: 0.48–0.72), cancer-specific survival (HR: 0.40, 95% CI: 0.27–0.61), and disease-free survival (HR: 0.72, 95% CI: 0.57–0.91).[15]

The circulating lymphocyte also has a role as a prognostic factor. The low pre-treatment lymphocyte-to-monocyte Ratio (LMR) group had a significantly worse overall survival rate ($p = 0.0011$). Moreover, patients who demonstrated low pre-treatment LMR and normalization after treatment exhibited a better overall survival rate than the patients with low pre- and post-treatment LMR values. Lymphocytes play an important role in the antitumor immunity of the host, including cytotoxic cell death and the inhibition of tumor cell proliferation and migration. On the other hand, monocytes play an important role in tumor progression and metastasis. Tumor-associated macrophages (TAMs), which are derived from circulating monocytes, suppress adaptive immunity and promote angiogenesis, invasion, migration, and tumor growth.[16] Significantly worse disease-free survival can be observed in patients with lower LMR levels (<3.78) using uni- and multi-variate analyses ($p = 0.01$ and $p = 0.015$, respectively).[17] The relationship between circulating lymphocyte and infiltrating lymphocyte in colorectal cancer is reported by Pane et al. (2015).[18] Neutrophil-to-lymphocyte ratio (NLR) of

5 or more results in significantly worse prognosis, higher pT- and pN-stage, and a greater incidence of extramural venous invasion compared to NLR below 5; meanwhile, moderate-to-severe presence of infiltrating lymphocytes in tumor margin is a significantly better prognosis compared to total absence or a mild presence. Infiltrating lymphocyte is also significantly higher in patients with NLR below 5. In this study, the circulating lymphocyte was not measured, and its impact on survival was not evaluated thus. The PNL was increasing the infiltrating of the rectosigmoid cancer; therefore, on the basis of Pane's study results,[18] we assume that PNL will also increase the circulating lymphocyte and could enhance the survival rate. Further study is needed to elaborate our hypotheses.

Since our study had only a one-group pre- and post-test design, the history and maturity factor may affect the results of the study. This is the main limitation of this study. Therefore, the next study should be randomized control trial (Phase III clinical trial) that makes maturity and history factors comparable and individual effect of the PNL extract can be measured. However, results of this study support the potential role of PNL as an immune modulator in the treatment of rectosigmoid cancer, but further research should be carried out to study in depth the role of PNL extract in arresting the cancer cell growth. In conclusion, it is clear that PNL extract improves the infiltrating lymphocyte and increases the apoptosis index, partly through perforin-granzyme-B pathway in patients with rectosigmoid cancer.

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