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Anticancer Activity of *Phyllanthus Niruri* Linn Extract in Colorectal Cancer Patients: A phase II Clinical Trial

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ABSTRACT

Phyllanthus niruri Linn (PNL) is a folk medicine that believed to exhibit anticancer effect. Granzyme, one of serine proteases produced by cytotoxic-T-lymphocytes (CTL) and natural killer cells (NK cells), is suggested as a prognostic marker of cancer. This study aimed to assess the effect of PNL extract on colorectal cancer cells growth by measuring granzyme expression. An experimental study utilized pre- and post-treatment design was conducted in Dr. Kariadi General Hospital, Semarang, Indonesia from May to July 2016. Hospitalized colorectal cancer patients were administrated with daily oral PNL extract for 14 days. Granzyme expressions before and post-treatment were measured using immunohistochemical staining technique. The difference of granzyme expression was analyzed using paired t-test. Fifteen patients with colorectal cancer were enrolled in this study. The mean granzyme expression of pre- and post-treatment was 25.46%±4.82% and 65.71%±7.91%, respectively. There was a significant increase of granzyme expression in post-treatment compared to pre-treatment group, $p<0.001$. In conclusion, PNL extract increased granzyme expression on colorectal cancer patients, suggesting its role as anticancer agent.

Keywords: *Phyllanthus niruri* Linn, granzyme expression, colorectal cancer, PNL, anticancer

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INTRODUCTION

Colorectal cancer is one of the deadliest cancers globally, ranked the fifth cause of cancer-related death in 2018.[1] More than 2.7 million people worldwide suffered from colorectal cancer, made it is the third most prevalent cancer after breast and prostate cancer.[1] Surgery remains the most effective treatment for localized and early stage colorectal cancer, while adjuvant radio and chemotherapy following surgery are strongly recommended for later stage.[2] However, chemotherapy and radiation showed low efficacy and high recurrence rate on colorectal cancer.[3, 4] Approaches have been taken to search for alternative cancer drugs from plant sources due to their low toxicities and costs, led to the discovery of many novel anticancer drugs such as vinka alkaloids, vinblastine and vincristine, taxol, camptothecins, and podophyllotoxins.[3, 5]

Immune system is suggested to play an important role in cancer cells development. Tumor induces specific adaptive immune response through infiltration of lymphocytes and Natural Killer (NK) cells into tumor cells.[6] Among them, cytotoxic T lymphocytes (CLTs) play an important role in cancer prognostic and clinical management.[7] High tumor-infiltrating CTLs in colorectal tumors patients was associated with significantly decreased mortality.[8] A previous study also found inversed association between CTL infiltration in tumor cells and the risk of colorectal cancer recurrence or mortality.[9] Granzymes, one of serine proteases, is mainly expressed by CTL and NK cells as the immune response against cells transformation.[10, 11] Although granzyme A, B, H, and M are identified in human, granzyme B is the most abundant (up to 70% of total granzymes) and the most potent.[11] Some granzymes exert cytotoxicity effects by inducing tumor or virus-infected cells death after being delivered intracellular by perforin.[12, 13] Granzyme B exhibits cytotoxic activity against cancer cells and is suggested as a positive prognostic marker in human colorectal cancer.[14] CTL infiltration in tumor cells and higher granzyme B expression were significantly associated with lower risk of colorectal cancer-associated death, even after considering the stage and other confounders.[7]

Immunomodulators are promising treatment to improve survival and reduce the recurrence of colorectal cancer.[15] One of the potent immunomodulator is *Phyllanthus niruri* Linn (PNL), a member of the *Euphorbiaceae*, which has been used as traditional medicine for decades in many countries, including Indonesia.[15, 16] A previous study showed that PNL increased phagocytosis and macrophage chemotaxis, neutrophil chemotaxis, NK cells cytotoxicity, and complementary hemolysis activities.[17] PNL also increased lymphocytes T cells proliferation by increasing TNFα, IFNγ, IL-4, IgM and IgG, and decreased IL-2 and IL-10.[17]

Studies in the recent years showed that PNL exhibits anticancer,[18] antiviral,[19] antioxidant,[20] anti-inflammatory[21] and antidiabetic activities,[22] and protection against radiation.[23] In vitro study showed that PNL inhibited the development of experimental colorectal cancer on Sprague-Dawley male rat through perforin-granzyme pathway and significantly decreased cancer cells' proliferative marker AgNORs and macroscopic tumor growth.[17] Spray-dried extracts of PNL followed by cisplatin showed a significant cytotoxic effect on colorectal cancer cells in another in vitro study.[24] A study on hepatic carcinoma cell showed that PNL increased apoptosis and reduced the viability of liver cancer cells.[18] A phase II clinical trial on rectal cancer patients showed that PNL extract significantly increased cancer cell apoptosis and

infiltrating lymphocyte, granzyme-B, perforin, and caspase-3 expression.[15]

The overmentioned studies[15, 17, 18, 24] implied that PNL is a promising treatment for colorectal cancer. However, most studies were conducted in vitro and used animal experimental models. Therefore, we conducted a study on colorectal cancer patients to further evaluate the effect of PNL extract on the growth of cancer cells through perforin-granzyme pathway by measuring granzyme expression.

MATERIAL AND METHODS

Ethical approval

The study has been approved by the Ethical Clearance Committee of Dr. Kariadi General Hospital - Diponegoro University, Semarang, Indonesia. Written consent was obtained from all patients prior to the study.

Study setting and patients

A pre- and post- study design with one group of colorectal cancer patients was employed to examine the expression of granzyme, as indicator of cancer cell growth's inhibition, after PNL administration. The study was conducted at Dr. Kariadi General Hospital, Semarang, Indonesia from May to July 2016. Hospitalized patients with resectable non-obstructive colorectal cancer, aged ≥40 years old, BMI ≥20 kg/m^2, hemoglobin ≥10%, and had undergone abdominal multislice computed tomography (MSCT) with intravenous contrast were recruited in this study.

Study procedure

All patients enrolled in this study underwent colonoscopy biopsy. The specimens from biopsy were further processed and examined to determine the granzyme expression of pre-treatment. The patients were then treated with oral 100mg PNL extract Stimuno® daily for 14 days; started from the next day after colonoscopy biopsy. Stimuno® contains of a single formula of PNL extract and has been approved by Indonesian Food and Drug Administration as a phytopharmaca since 2005.[25] Tumor resection was carried out after 14 days of treatment and tumor specimens were later examined to determine granzyme expression for post-treatment.

Expression of granzyme in tumor cells was measured using immunohistochemical staining. Briefly, after the specimens were fixed with formalin and embedded into paraffin block, immune histochemical staining was conducted for detecting total granzyme of cancer cells. The expression of granzyme was measured under 400x magnification by two independent anatomical pathologists. Microscopic diagnosis and immune histochemical staining were conducted at the Anatomical Pathology Laboratory of Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. For each patient, 500 tumor cells were counted and the percentage of tumor cells positive granzyme were calculated for each 100 cells (i.e. there were five percentage of granzyme for each patients). The percentage of granzyme was defined as number of tumor cells positive granzyme divided by 100 tumor cells.

Statistical analyses

Data of percentage of granzyme expression were presented in mean, standard deviation (SD), and median. The normality of the data was assessed using Shapiro–Wilk test. Paired t-test was employed to compare the expression of granzyme pre- and post-treatment. Significance was defined if p-value ≤0.05 and statistical analysis was carried out with SPSS ver.17.0 for Windows.

RESULTS

Fifteen patients with colorectal cancer were enrolled in this study. The median expression of granzyme of each patient from both groups are presented in Table 1. Prior treatment, the highest and the lowest percentage of granzyme expression was 34.36% (min and max: 17.43-51.69%) and 16.60% (10.21–23.56%), respectively. Meanwhile, the highest and the lowest median of granzyme expression in post-treatment group was 80.09% (71.11-86.09%) and 49.75% (37.08–65.64%), respectively (**Table 1**). The median of granzyme expression was 24.93% (16.60–34.36%) for pre-treatment and 68.15% (49.75–80.09%) for post-treatment. Overall, the expression of granzyme after PNL treatment was higher compared to pre-treatment.

Table 1. Granzyme expression of each colorectal cancer patient pre- and post-treatment with *Phyllanthus niruri* Linn (PNL)

Patient ID	Granzyme expression (%), median (min-max)	
	Pre-treatment	Post- treatment
A	24.93 (15.31 – 30.45)	59.65 (37.55 – 81.63)
B	20.03 (13.79 – 33.16)	49.75 (37.08 – 65.64)
C	26.28 (15.19 – 53.50)	70.77 (54.92 – 83.72)
D	16.60 (10.21 – 23.56)	70.69 (60.00 – 81.43)
E	29.73 (13.06 – 49.13)	67.62 (37.61 – 88.11)
F	21.49 (9.46 – 28.00)	68.96 (60.90 – 86.23)
G	20.33 (15.65 – 31.90)	80.09 (71.11 – 86.09)
H	23.61 (17.79 – 28.57)	69.16 (55.67 – 86.52)
I	34.36 (17.43 – 51.69)	70.71 (55.05 – 84.09)
J	29.94 (14.00 – 49.43)	68.14 (38.68 – 88.65)
K	24.44 (18.63 – 31.68)	71.29 (55.21 – 83.95)
L	29.38 (12.63 – 48.84)	67.27 (37.16 – 87.98)

M	15.66 (32.84 – 42.70)	60.29 (39.80 – 76.26)
N	25.56 (18.59 – 34.84)	53.93 (39.59 – 70.60)
O	23.95 (15.32 – 29.41)	57.37 (46.61 – 70.39)
Median	24.93 (16.60 – 34.36)	68.15 (49.75 – 80.09)

The mean granzyme expression of pre- and post-treatment was 25.46%±4.82% and 65.71%±7.91%, respectively. Individual mean expression of granzyme are shown on **Figure 1**. Shapiro–Wilk test suggested that the data of both groups were distributed normally with p=0.978 and p=0.198 for pre- and post-treatment group, respectively and therefore paired t-test was employed. Paired t-test revealed that granzyme expression was significantly higher after PNL treatment (p<0.001).

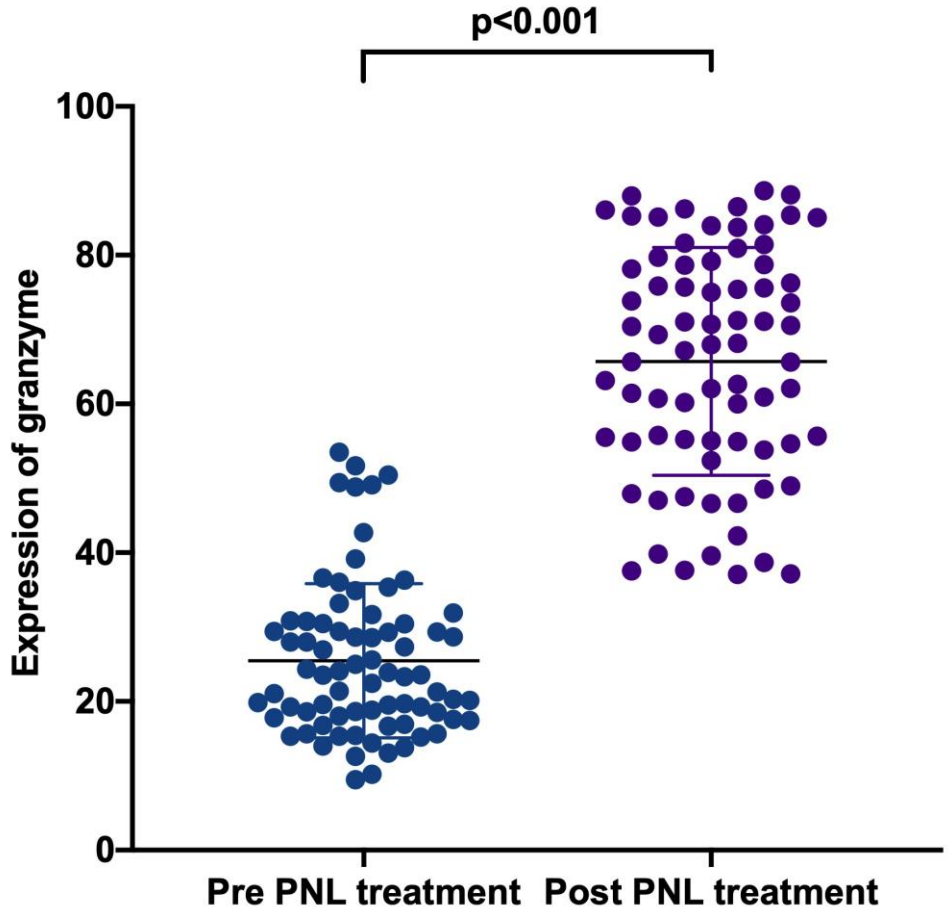

Figure 1. Individual granzyme expression of each colorectal cancer patient pre-treatment and post-treatment with oral 100mg *Phyllanthus niruri* Linn (PNL) extract daily for 14 days.

DISCUSSION

A previous study showed that PNL extract exhibited anticancer activity and had low toxicity.[16] In vitro study also found that PNL extract inhibited the growth of colorectal cancer cells on experimental rats.[17] Thus, we undertaken this Phase II Clinical Trial to assess the effect of PNL extract on human colorectal cancer.

Our data found that PNL extract significantly increased granzyme expression. The finding is similar to a previous study that showed PNL extract significantly increased the expression of infiltrating lymphocyte, granzyme-B, perforin, and caspase-3 in recto sigmoid cancer patients.[15] The role of PNL extract as an apoptotic agent in this study is probably through perforin-granzyme pathway.[26] Granzyme A and B are important granzymes in apoptosis mechanism. Granzyme B pathway converges on the cleavage of caspase-3 as apoptosis executor, along with other executors of apoptosis like caspase-6 and -7. Meanwhile, granzyme A pathway activates a parallel, caspase-independent cell death pathway, facilitating single-stranded DNA breaks.[11, 26] The association between granzyme expression and colorectal cancer has been studied previously. Granzyme B was suggested as positive prognostic marker in colorectal cancer, where low expression of granzyme B was associated with early signs of colorectal cancer metastasis.[14] Higher granzyme B expression and CTL infiltration in tumor cells were significantly associated with lower risk of colorectal-associated death.[7]

The result of this study provides additional evidence to previous studies that suggest the promising effect of PNL as anticancer.[16-18, 24] A study showed that PNL extract could arrest hepatic carcinoma cell line, had antitumor potential, and had lower toxicity in normal cells.[16] Another

study showed that spray-dried extract of PNL increased apoptosis and reduced liver cancer cells viability.[24] Particularly for colorectal cancer, a study on Wistar rat showed that PNL inhibited the development of colorectal cancer cells and significantly decreased tumor growth and cancer cells' proliferative marker AgNORs.[17] The administration of spray-dried extract of PNL combined with cisplatin showed a significantly better cytotoxic effect on colorectal cancer cells compare to spray-dried extract of PNL or cisplatin alone.[24] The result of this study supports the potential role of PNL as anticancer agent for colorectal cancer treatment.

This clinical trial was conducted on one group and did not consider history and maturity factors of colorectal cancer cells, which might affect the results. A randomized clinical trial needs to be carried out in the future to compare maturity and history of colorectal cancer, and to measure individual effect of the PNL extract. Moreover, the use of crude extract of PNL in this study made it impossible to identify the active component that increased granzyme expression. A study utilizing a purified extract of PNL is warranted to further investigate the bioactive component. However, this study still able to elucidate the potential role of PNL as immunomodulator for colorectal cancer treatment.

CONCLUSION

Administration of oral daily 100mg PNL extract for 14 days increased granzyme expression in colorectal cancer patients suggesting its potential anticancer activities. This study highlights the possibility to carry out a randomized clinical trial to assess the effect of PNL extract on clinical manifestation or disease progression of colorectal cancer.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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REFERENCE

1. WHO. Cancer Today: Population Fact Sheets. World Health Organization, 2020.
2. NCCN. Clinical Practice Guidelines in Oncology (NCCN Guidelines) : Colon Cancer Version 3. National Comprehensive Cancer Network, 2015.
3. Ramasamy S, Wahab NA, Abidin NZ, Manickam S, Zakaria Z. Growth Inhibition of Human Gynecologic and Colon Cancer Cells by Phyllanthus watsonii through Apoptosis Induction. PloS ONE 2012; 7.
4. Santiago L, Castro M, Sanz-Pamplona R, Garzon M, Ramirez-Labrada A, Tapia E, et al. Extracellular Granzyme A Promotes Colorectal Cancer Development by Enhancing Gut Inflammation. Cell Rep 2020; 32.
5. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. J Ethnopharmacol 2005; 100:72-9.
6. Waldner M, Schimanski CC, Neurath MF. Colon cancer and the immune system: The role of tumor invading T cells. World J Gastroenterol 2006; 12:7233-38.
7. Prizment AE, Vierkant RA, Smyrk TC, Tillmans LS, Nelson HH, Lynch CF, et al. Cytotoxic T-cells and granzyme B associated with improved colorectal cancer survival in a prospective cohort of older women. Cancer Epidemiol Biomarkers Prev 2017; 26:622–31.
8. Mei Z, Liu Y, Liu C, Cui A, Liang Z, Wang G, et al. Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis. Br J Cancer 2014; 110:1595-605.
9. Nosho K, Baba Y, Tanaka N, Shima K, Hayashi M, Meyerhardt JA, et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. J Pathol 2010; 222:350-66.
10. Mulder WM, Bloemena E, Stukart MJ, Kummer JA, Wagstaff J, Scheper RJ. T cell receptor-zeta and granzyme B expression in mononuclear cell infiltrates in normal colon mucosa and colon carcinoma. Gut 1997; 40:113-9.
11. Trapani J. Granzymes: A Family of Lymphocyte Granule Serine Proteases. Genome biology 2001; 2.
12. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. Annu Rev Immunol 2008; 26:389-420.
13. Bovenschen N, Kummer JA. Orphan granzymes find a home. Immunol Rev 2010; 235:117-27.
14. Salama P, Phillips M, Platell C, Iacopetta B. Low expression of Granzyme B in colorectal cancer is associated with signs of early metastastic invasion. Histopathology 2011; 59:207-15.
15. Riwanto I, Budiono P, Mughni A, Martahadinan, Putra ND, Mambu TDB, et al. Phyllanthus niruri Linn increase infiltrating lymphocyte and apoptosis of rectosigmoid cancer patients: A Phase II Clinical Trial. Bali Med J 2017; 6:509-13.
16. Zheng Z-Z, Chen L-H, Liu S-S, Deng Y, Zheng G-H, Gu Y, et al. Bioguided Fraction and Isolation of the Antitumor Components from Phyllanthus niruri L. Biomed Res Int 2016.
17. Sawitri E, Riwanto I, Tjahjono T, Dharmana E. [Effect of Phyllanthus niruri Linn extract on tumor growth and cell proliferation of colorectal cancer: experimental study in 1.2 DMH-induced Sprague-Dawley rats]. M Med Indones 2012; 46:25-32.
18. Júnior RFdA, Souza TPd, Pires JGL, Soares LAL, Araújo AAd, Petrovick PR, et al. A dry extract of Phyllanthus niruri protects normal cells and induces apoptosis in human liver carcinoma cells. Exp Biol Med 2012; 237:1281-8.
19. de-Melo MN, Soares LA, Porto CR, de-Araújo AA, Almeida Md, de-Souza TP, et al. Spray-dried extract of Phyllanthus niruri L. reduces mucosal damage in rats with intestinal inflammation. J Pharm Pharmacol 2015; 67:1107-18.
20. Sailaja R, Setty OH. Protective effect of Phyllanthus fraternus against allyl alcohol-induced oxidative stress in liver mitochondria. Journal of Ethnopharmacology 2006; 105:201-9.
21. Lai CH, Fang SH, Rao YK, al e. Inhibitionof Helicobacter pylori-induced inflammation in human gastric epithelial AGS cells by Phyllanthus urinaria extracts. Journal of Ethnopharmacology 2008; 118:522-6.
22. Okoli CO, Obidike IC, Ezike AC, Akah PA, Salawu OA. Studies on the possible mechanisms of antidiabetic activity of extract of aerial parts of Phyllanthus niruri. Pharm Biol 2011; 49:284-55.

23. Londhe JS, Devasagayam TPA, Foo LY, Ghaskadbi S. Radioprotective Properties of Polyphenols from Phyllanthus amarus Linn. Journal of Radiation Research 2009; 50:303-9.
24. Júnior RFdA, Soares LAL, Porto CRdC, Aquino RGFd, Guedes HG, Petrovick PR, et al. Growth inhibitory effects of Phyllanthus niruri extracts in combination with cisplatin on cancer cell lines. World J Gastroenterol 2012; 18:4162-8.
25. JamuDigital. [Stimuno: Indonesian phytoprarmaca that succeed in global market]. JamuDigital.com, 2019.
26. Elmore S. Apoptosis : a review of programmed cell death. Toxicol Pathol 2007; 35:495-516.