

Partial Purification of Cytotoxic Peptides against Gastric Cancer Cells from Protein Hydrolysate of *Euphorbia hirta* Linn.

S. Yodyingyong, C. Chaichana, C. Nuchsuk, S. Roytrakul, N. P. T-Thienprasert, S. Ratanapo

Abstract—Protein hydrolysates prepared from a number of medicinal plants are promising sources of various bioactive peptides. In this work, proteins from dried whole plant of *Euphorbia hirta* Linn. were extracted and digested with pepsin for 12h. The hydrolysates of lesser than 3 kDa were fractionated by a cut-off membrane. The peptide hydrolysate was then purified by an anion-exchange chromatography on DEAE-Sephacel™ column and reverse-phase chromatography on Sep-pak C₁₈ column, respectively. The cytotoxic effect of each peptide fraction against a gastric carcinoma cell line (KATO-III, ATCC No. HTB103) was investigated using colorimetric MTT viability assay. A human liver cell line (Chang Liver, CLS No. 300139) was used as a control normal cell line. Two purified peptide peaks, peak I and peak II at 100 µg peptides mL⁻¹ affected cell viability of the gastric cancer cell lines to 63.85±4.94 and 66.92±6.46%, respectively. Our result showed for the first time that the peptide fractions derived from protein hydrolysate of *Euphorbia hirta* Linn. have anti-gastric cancer activity, which offers a potential novel and natural anti-gastric cancer remedy.

Keywords—Cytotoxic, peptides, *Euphorbia hirta* Linn., gastric carcinoma.

I. INTRODUCTION

GASTRIC cancer ranks the fourth among commonly diagnosed malignancy and is the second leading cause of cancer-related deaths worldwide [1]. Although surgery is an effective treatment for the majority of patients, patients with advanced cancer are primarily treated with drugs [2] that have adverse side effects and toxicity complications. Ineffective chemotherapy against the gastric cancer in some patients also occurs due to multidrug resistance [3], [4]. To reduce the adverse effects and reverse the multi-drug resistant, researches on cancer preventive peptides have been carried out with great interest.

Euphorbia hirta Linn. (*E. hirta*, commonly called snake grass) is a medicinal, rhizomatous herb distributed in tropical

S. Yodyingyong, C. Chaichana, C. Nuchsuk, and N.P. T-Thienprasert are with the Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand (phone: 66-2-5625555; fax: 66-2-5614627; e-mail: khao_takiab@hotmail.com, cctomachi52@gmail.com, pui.chanthakan@gmail.com, fscinnp@ku.ac.th).

S. Roytrakul is with National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Pathum Thani 12120, Thailand (phone: 66-2-5646700; fax: 66-2-5646707; e-mail: sittiruk@biotec.or.th).

S. Ratanapo is with the Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand (corresponding author; phone: 66-2-5625555; fax: 66-2-5614627; e-mail: fscisnr@ku.ac.th).

countries. The plant extract has been reported as repellent, antifeedant, antibacterial, and against worms [5], [6]. In Thailand, the plant is commonly called “Nom-raatchasee, Phak-khom-daeng Yaa-nam-muek, Yaa-lang-ueng” and its whole plant extract has long been used in folk medicine to treat asthma, coughs, diarrhea and amoebic dysentery. For pharmaceutical purposes, most of studies aim to identify new antibiotic drugs obtained from organic-solvent extraction of medicinal plants, but a few studies have focused on plant peptides. In this study, protein hydrolysates from *E. hirta* were produced by pepsin hydrolysis. This enzymatic hydrolysis mimicked the production of protein hydrolysate from human gastric digestion. A peptide with free-radical scavenging activity and cytotoxicity against a human-gastric carcinoma cell line was then isolated and evaluated for its potency.

II. MATERIALS AND METHODS

A. Medicinal Plants and Preparation of Protein Hydrolysate

Dried whole plant of *E. hirta* was purchased from a local market in Bangkok, Thailand. It was cut into small pieces and grounded by a blender. The protein was extracted by stirring with 0.01% (w/v) sodium dodecyl sulfate (SDS) at room temperature for 12h and then dialyzed against 50mM Tris-HCl buffer pH 7.5 overnight at 4°C. After sonication for 15 min, the suspension was centrifuged at 10,000 x g at room temperature for 30min. The supernatant was then collected, and its pH was adjusted to 3.5 with 6 M HCl. The supernatant was then hydrolyzed by pepsin (Sigma-Aldrich) at an enzyme to protein ratio of 1:25 (mg/mg protein) at 37°C for 12h. The reaction was stopped by boiling for 10min. The supernatant was collected by centrifugation at 10,000 x g at room temperature for 10min and stored at 4°C.

B. Purification of Peptides by Anion Exchange Chromatography

The protein hydrolysate of *E. hirta* was applied to a DEAE Sephacel™ (GE Healthcare, Little Chalfont, UK) column (1.5 × 20cm) which was equilibrated with 50mM Tris-HCl, pH 7.5. The unbound proteins were washed out with the same buffer at a flow rate of 40mL h⁻¹. The absorb proteins were eluted stepwise with 0–2 M NaCl in the Tris-HCl buffer. Fractions of 4mL were collected and the UV absorption was monitored at 280nm. Each fraction was determined for cytotoxicity against the gastric carcinoma cell lines and the active fractions were pooled, dialysed against the Tris-HCl buffer, and kept at 4°C.

C. Purification of Peptides by Reverse-Phase HPLC

The pool active fraction from DEAE-Sephacel™ column was separated by a reverse-phase HPLC using a Delta-Pak C18 column (100 Å, 3.9mm x 150mm, Interlink Scientific Services Ltd.) previously equilibrated with 0.1% trifluoroacetic acid (TFA). The column was eluted stepwise with 6% acetonitrile in 0.1% TFA for 10min and 60% acetonitrile in 0.1% TFA for 10min at a flow rate of 1 mL min⁻¹. Fractions were collected at 1min intervals, and the elution peaks were detected at 220nm. Each fraction was determined for cytotoxicity against the gastric carcinoma cell lines.

D. In vitro Cytotoxicity Assay

Cytotoxic effect of the purified peptide from *E. hirta* against human a gastric cancer and a normal cell lines were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay [7], based on inhibition of the cellular conversion of a tetrazolium salt into a colored formazan product. The human gastric carcinoma (KATO-III, ATCC No. HTB103) and a normal liver (Chang Liver, CLS No. 300139) 1 × 10⁴ cells were seeded into each well of 96-well tissue culture plates. After 24h incubation at 37°C in a humidified 5% CO₂ atmosphere, the culture was added with 200µl of the purified protein dilutions per well. For cell controls, 200µL of medium without the purified protein was added. After 24h at 37°C in a humidified 5% CO₂ atmosphere, the medium was removed and 100µL of MTT (0.35 mg. mL⁻¹) solution was added to each well; the plates were then incubated for 4h at 37°C. The MTT solution was removed and 200µL of DMSO was added to each well to dissolve formazan crystals. The absorbance (A) was read by a microplate reader at 570nm. The percentage of cell viability was calculated as following: % cell viability = {(A treated cell – A blank) / (A control – A blank)} × 100; where control is only untreated cells and blank is only DMSO. Results were expressed as the means ± the standard deviations.

E. Assay of DPPH Radical Scavenging Activity

DPPH radical scavenging activity of the peptides was measured using the method described by Choi et al. [8]. Briefly, a 1mL of a 0.3mM DPPH in ethanol solution was added to 2.5mL of peptide solution. After incubation at room temperature for 30 min, the absorbance was performed at 518 nm. Ethanol instead of DPPH was used for the blank, while distilled water instead of sample was used for the control.

F. Protein and Peptide Determination

Protein and peptide concentration was determined according to the method of Lowry et al. [9] using bovine serum albumin as the standard.

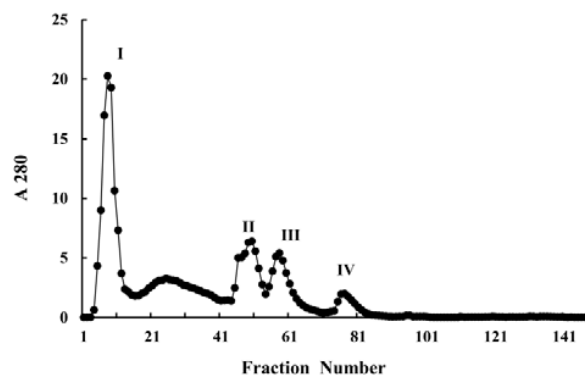


Fig. 1 Elution profile of peptides from a DEAE-Sephacel™ column. The lesser than 3 kDa protein hydrolysate of *E. hirta* Linn. (2mg peptide mL⁻¹, 10ml) was applied to the column (2x2cm), washed with 20mM sodium acetate buffer, pH 4.5 and eluted step-wise with the buffer containing NaCl at 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 M, at flow rate 40mL h⁻¹. Fractions (2mL each) were collected and pooled. Fractions no. 5-13 (peak I), no. 46-52 (peak II), no. 55-61 (peak III) and no. 74-80 (peak IV) were pooled

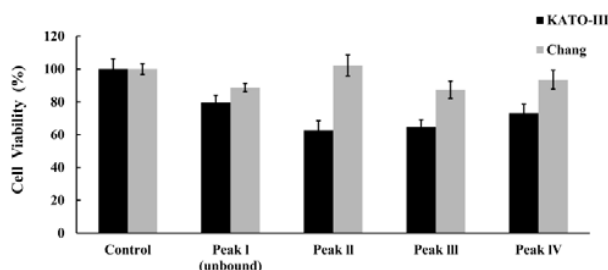


Fig. 2 The cytotoxic effects of DEAE-Sephacel™ peptide fractions from *E. hirta* Linn against the human gastric carcinoma cell line (KATO-III, ATCC No. HTB103) and the control normal liver cell line (Chang Liver, CLS No. 300139). The results are shown as the mean percentage of viability of cells ± SD. The experiment was performed two times in triplicate (n=3)

III. RESULTS

After SDS extraction, the total proteins from *E. hirta* was digested with pepsin and ultrafiltered to obtain the hydrolysates of lesser than 3 kDa. Anion-exchange chromatography of the protein hydrolysate was subsequently performed. As a result, a large peak of unbound fractions (peak I) and three separated peaks of bound fractions (peak II, III and IV) were yielded (Fig. 1). Each pooled peak was tested for its effect on the cell viability of a human gastric carcinoma cell line (KATO-III) by using a normal cell line (Chang) as a control. Over the same concentration tested (0.2mg mL⁻¹), peak II and peak III exerted the moderate cytotoxic effects against the KATO-III cell line with cell viability at 62.77±5.82 % and 64.68±4.62 %, respectively (Fig. 2). Antioxidant activity of each peptide peak was determined by DPPH radical scavenging activity as depicted in Table I. The peptide peak III showed the highest radical scavenging activity with about 2.5 and 2.7 times higher than the peptides peak II and peak IV, respectively. Therefore, the peptide peak III was chosen to be

further purified by reverse-phase chromatography on a Sep-Pak C₁₈ column. As shown in Fig. 3, four peptide peaks were isolated and named consecutively I-IV, among which, peak I and II were identified to be moderate cytotoxic peptides against the Kato III cell lines with 40% inhibitory effect (Fig. 4).

TABLE I
DPPH* SCAVENGING ACTIVITY OF DEAE-SEPHACEL™ PEPTIDE FRACTIONS FROM *E. HIRTA* LINN. (N = 3)

Fraction	Average Peptide (mg mL ⁻¹)	Scavenging activity (%)
Peak I (unbound)	1.87±0.10	99.70±0.60
Peak II	0.14±0.04	36.46±1.80
Peak III	0.13±0.03	92.89±0.69
Peak IV	0.10±0.02	34.77±1.13

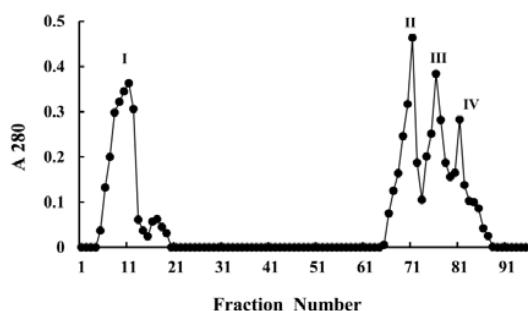


Fig. 3 Elution profile of peptides from a Sep-pak C₁₈ column. An aliquot of peak II (0.2mg peptide ml⁻¹ 1mL, pH =7) obtained from DEAE-Sephadex™ chromatography was applied to a Sep-pak C₁₈ column (500µL) pre-equilibrated with solution A (98% MilliQ-H₂O, 2% CH₃CN, 0.1% FA) 10mL and solution B (65% CH₃CN, 35% MilliQ-H₂O, 0.1% FA) 5mL. The elution was carried out at flow rate of 1mL min⁻¹ with 0-60% acetonitrile. Fractions (1mL each) were collected and pooled

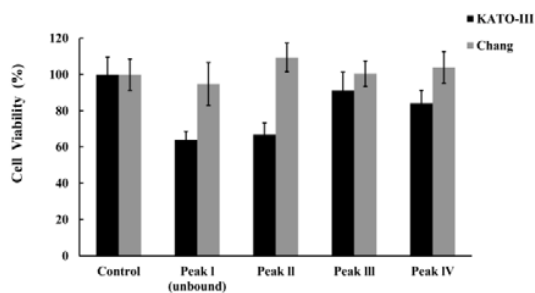


Fig. 4 The cytotoxic effects of Sep-pak C₁₈ peptide fractions from *E. hirta* Linn against the human gastric carcinoma cell line (KATO-III, ATCC No. HTB103) and the control normal liver cell line (Chang Liver, CLS No. 300139). The results are shown as the mean percentage of viability of cells ± SD. The experiment was performed two times in triplicate (n=3)

IV. DISCUSSION

Various plant and animal proteins have the ability to exert physiological benefits in human beings by its certain amino acid sequences. The amino acid sequences remain inactive when they are present as part of the continuous primary

structure of the parent protein. However, when the parent protein is hydrolysed by an appropriate proteolytic enzyme, the peptide is released in active form and exerts biological effects [10]. The most studied plant derived bioactive peptides are the anti-hypertensive and antioxidant peptides [11], [12], but few are anticancer peptides [13]. In this study, protein hydrolysate from *E. hirta* was prepared by hydrolysis of the plant proteins with pepsin. As most bioactive peptides contain 2–20 amino acid residues per molecule and have low molecular weight, the protein hydrolysate of *E. hirta* was therefore membrane ultrafiltered to obtain the low molecular weight peptides of lesser than 3 kDa. The small low molecular weight peptides have been reported to have high biological effects because their chance to cross the intestinal barrier and exert biological effects [14]-[16]. A number of antioxidants have been postulated to play a role in the prevention and treatment of certain cancers [17]. Based on our result, partially purified peptide with lesser than 3 kDa of *E. hirta* possessed excellent antioxidant (free radical scavenging) activity and cytotoxicity against a gastric carcinoma cell line. This peptide could have potential nutraceuticals against gastric cancer.

ACKNOWLEDGMENTS

This work was supported by the Kasetsart University Research and Development Institute (KURDI) and partly supported for the contribution by Faculty of Science, Kasetsart University. We thank Assistant Professor Dr. Kiattawee Choowongkomol for providing the plant sample.

REFERENCES

- [1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics", *CA Cancer J. Clin.*, vol. 61, pp. 69–90, 2011.
- [2] H. Lage, "An overview of cancer multidrug resistance: a still unsolved problem", *Cell Mol. Life Sci.*, vol. 65, pp. 3145–3167, 2008.
- [3] D. Zhang, and D. Fan, "New insights into the mechanisms of gastric cancer multidrug resistance and future perspectives", *Future Oncol.*, vol. 6, pp. 527–537, 2010.
- [4] D. Fan, X. Zhang, X. Chen, Z. Mou, J. Hu, S. Zhou, J. Ding, and K. Wu, "Bird's-eye view on gastric cancer research of the past 25 years", *J. Gastroenterol. Hepatol.*, vol. 20, pp. 360–365, 2005.
- [5] H. M. Adamu, O. J. Abayeh, M. O. Agho, A. L. Abdullahi, A. Uba, H. U. Dukku, and B. M. Wufem, "An ethnobotanical survey of Bauchi State herbal plants and their antimicrobial activity". *J. Ethnopharmacol.*, vol. 99, pp. 1-4, 2005.
- [6] J. Parekh, and V. S. Chanda, "In vitro antimicrobial activity and phytochemical analysis of some Indian medicinal plants." *Turk. J. Biol.*, vol. 31, pp. 53-58, 2007.
- [7] T. Mossmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." *J. Immunol. Methods*, vol. 65, pp. 55-63, 1983.
- [8] C. W. Choi, S. C. Kim, S. S. Hwang, B. K. Choi, H. J. Ahn, M. Y. Lee, S. H. Park, and S. K. Kim, "Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison." *Plant Science*, vol. 163, pp. 1161-1168, 2002.
- [9] O. H. Lowry, N. J. Rosebrough, A. I. Fan, and R. J. Randall, "Protein measurement with the folin phenol reagent." *J. Biol. Chem.*, vol. 193, pp. 265-275, 1951.
- [10] R. Hartmann, and H. Meisel, "Food-derived peptides with biological activity from research to food applications." *Curr. Opin. Biotechnol.*, vol. 18, pp. 163-169, 2007.
- [11] R. E. Aluko, "Antihypertensive properties of plant-derived inhibitors of angiotensin I-converting enzyme activity: A Review." *Recent Prog. Med. Plants*, J. N. Govil and V. K. Singh (eds.), vol. 22-Phytopharmacology and Therapeutic Values IV, pp. 541-561, 2008.

- [12] M. C. García, P. Puchalska, C. Esteve, and M. L. Marina, "Vegetable foods: A cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities. Review Article" *Talanta*, vol. 106, pp. 328-349, 2013.
- [13] S. J. Rayaprolu, N. S. Hettiarachchy, P. Chen, A. Kannan, and A. Mauromostakos. "Peptides derived from high oleic acid soybean meals inhibit colon, liver and lung cancer cell growth." *Food Res. Inter.*, vol. 50, 282, 2013, DOI: 10.1016/j.foodres.2012.10.021
- [14] Y. Y. Ma, Y. L. Xiong, J. J. Zhai, H. N. Zhu, and T. Dziubla. "Fractionation and evaluation of radical scavenging peptides from in vitro digests of buckwheat protein." *Food Chem.*, vol. 118, pp. 582-588, 2010.
- [15] N. S. Sampath Kumar, R. A. Nazeer, and R. Jaiganesh, "Purification and identification of antioxidant peptides from the skin protein hydrolysate of two marine fishes, horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*)." *Amino Acids*, vol. 42, 1641-1649, 2012.
- [16] N. S. S. Kumar, R. A. Nazeer, and R. Jaiganesh, "Purification and biochemical characterization of antioxidant peptide from horse mackerel (*Magalaspis cordyla*) viscera protein." *Peptides*, vol. 32, 1496-1501, 2011.
- [17] S. Wang, K. A. Meckling, M. F. Marcone, Y. Kakuda, and R. Tsao. "Can phytochemical antioxidant rich foods act as anti-cancer agents?" *Food Res. Inter.*, vol. 44, pp. 2545-2554, 2011.