Shortgun Proteomics Analysis of Mouse Colon Treated with *Eryngium fotidum* Linn. Extracts

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Abstract

Eryngium foetidum Linn.leaves (EF) have been traditionally used as a food and medicine in South East Asia. The average consumption of EF in Thai population in each age group is about 0.48 g/kg BW/day. EF was able to prevent colon carcinogenesis in mice treated with azoxymethane-dextran sulfate sodium. Moreover, there was no defect observed in colon tissues using a pathological approach. In the present study, the safety of EF at the translational level was investigated. Total proteins from 3 groups (Group 1; control group, Group 2; 0.8% EF and Group 3; 3.2% EF in diet) of each colon sample were analyzed by GeLC-MS/MS. After fractionation by SDS-PAGE, the proteins bands were divided according to their molecular weight followed by tryptic digestion and nanoLC-MS analysis. Protein quantitation and identification were determined by Decyder MS Differntial Analysis and Mascot softwares. The results showed significantly different levels of protein expression between EF-treated and non-treated mice. However, no significantly different expression was observed in 0.8% and 3.2% EF-treated mice for 15 weeks. No alteration of proteins involved in colon carcinogenesis was observed. In addition, pathological study did not showed any defect in colon tissues treated with EF. It can be concluded that the treatment with EF at 0.8% and 3.2% for 15 weeks in mice was considered as safe.

Keywords: Colon cancer, *Eryngium foetidum* Linn. leaves, Mouse, Proteomics

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กลไกการออกฤทธิ์ระดับโปรตีนด้วยเทคนิค shortgun โปรติโอมิกส์ของลำไส้ใหญ่ในหนูเม้าส์ที่ได้รับผักชีฝรั่ง

ฐิติพัฒน์ ลิขิตพฤกษ์ไพศาล[1], สิทธิรักษ์ รอยตระกูล[2], สุธาทิพย์ กิตติเสนาชัย[2], ปิยะ เต็มวิริยะนุกูล[3], ชนิพรรณ บุตรยี่[3*]

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บทคัดย่อ

ผักชีฝรั่ง (*Eryngium foetidum* Linn. leaves; EF) เป็นพืชสมุนไพรพื้นบ้านที่ชาวเอเชียตะวันออกเฉียงใต้นิยมนำมาบริโภคเป็นอาหารและมีสรรพคุณทางยา จากข้อมูลการบริโภคของประชากรไทยค่าเฉลี่ยของปริมาณการบริโภคผักชีฝรั่งเท่ากับ 0.48 กรัมต่อกิโลกรัมน้ำหนักตัวต่อวัน และมีรายงานการศึกษาพบว่าผักชีฝรั่งมีคุณสมบัติในเชิงป้องกันการเกิดมะเร็งลำไส้ใหญ่ในหนูเม้าส์ที่ถูกชักนำให้เกิดมะเร็งลำไส้จากสารก่อมะเร็ง azoxymethane และ dextran sulfate sodiumโดยไม่มีผลต่อการเปลี่ยนเปลี่ยนทางพยาธิวิทยาของเนื้อเยื่อบริเวณลำไส้ จึงเป็นที่มาของการศึกษาความปลอดภัยของการบริโภคผักชีฝรั่งในกลไกระดับโปรตีน โดยนำลำไส้ของหนูเม้าส์ในแต่ละกลุ่ม จากทั้งหมด 3 กลุ่ม (กลุ่มแรกได้รับอาหารปกติ กลุ่มที่สองเป็นกลุ่มที่ได้รับอาหารปกติร่วมกับผักชีฝรั่งในปริมาณร้อยละ 0.8 และกลุ่มที่สามเป็นกลุ่มที่ได้รับอาหารปกติร่วมกับผักชีฝรั่งในปริมาณร้อยละ 3.2) มาวิเคราะห์การแสดงออกของโปรตีนด้วยเทคนิค GeLC-MS/MS จากนั้นทำการแยกขนาดของโปรตีนด้วยเทคนิค SDS-PAGE และทำการสกัดโปรตีนออกจากแผ่นเจลด้วยวิธี trypsin digestion และทำการวิเคราะห์ด้วยเครื่อง nanoLC-MS โดยชนิดและปริมาณของโปรตีนจะถูกจำแนกความแตกต่างด้วยโปรแกรม Decyder MS และ โปรแกรม Mascot softwares จากการศึกษาครั้งนี้พบว่าปริมาณการแสดงออกของโปรตีนในกลุ่มควบคุมและกลุ่มทดลองที่ได้รับอาหารร่วมกับผักชีฝรั่งเป็นระยะเวลา 15 สัปดาห์ มีปริมาณที่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ แต่ผลในกลุ่มทดลองทั้งสองกลุ่ม (ส่วนผสม EF ร้อยละ 0.8 และส่วนผสม EF ร้อยละ 3.2) พบว่าปริมาณการแสดงออกของโปรตีนไม่มีความแตกต่างกันอย่างมีนัยสำคัญ อย่างไรก็ตามไม่พบการแสดงออกของโปรตีนในกลุ่มที่เกี่ยวข้องกับการก่อให้เกิดมะเร็งลำไส้ ซึ่งสอดคล้องกับผลทางพยาธิวิทยาที่ไม่พบการเปลี่ยนแปลงของเนื้อเยื่อลำไส้หนูเม้าส์ที่ได้รับอาหารร่วมกับผักชีฝรั่งเป็นระยะเวลา 15 สัปดาห์ สรุปได้ว่าการให้ผักชีฝรั่งที่ผสมอาหารปริมาณร้อยละ 0.8 และร้อยละ 3.2 แก่หนูเป็นระยะเวลา 15 สัปดาห์มีความปลอดภัย

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Introduction

A number of herbal products have been studied as anticancer agents[1]. The *Eryngium foetidum* Linn leaves (EF) also known as "Phakchi-farang" has been used as a traditional medicine for treatment of colds, fits, convulsions, fainting, headaches and malaria[2,3]. It has been found that EF contains essential nutrients and various phytochemicals, such as polyphenols, phenolic acids, terpenes, and alkaloids etc. The main chemical components of volatile oil in Thai EF are 3-dodecen-1-al (67.25%), dodecanal (7.49%), nor-copaanone (7.13%), and eugenol (4.30%)[4]. The health benefits of EF are mainly imposed due to antioxidant and anti-cancer properties. The antioxidant components of EF are Vitamin C, vitamin E, carotenoid, xanthophil, tannin, phenolic and flavonoid compounds[5]. There are several reports about the properties of EF in induction of cancer cell apoptosis. The phenolic compounds in volatile oil of EF showed the highest inhibition activity for human mammary carcinoma (MCF-7) (ED50 = 28.30 µg/ml)[4]. Other studies reported that the major components in EF (alpha-choloesterol, brassicasterol, campesterol and stigmasterol) had anti-inflammatory activity when studied on chronic and acute inflammation in mouse models[6].

Proteomics is the large-scale study of proteome to comprehensively map biological processes, such as the molecular mechanisms of carcinogenesis[7]. It should be noted that proteomics not only focus exclusively on the identification and quantification of these proteins, but also investigates their location, their modifications, their interactions and their functions[8].

Previously, the preventive and suppressive effects of EF were shown on colon carcinogenesis. EF (3.2% in diet) was able to prevent colon cancer in mice induced with azoxymethane (AOM) and dextran sodium sulfate (DSS) in comparison with control group[9]. No clastogenicity was found in mice after receiving 0.8%, 1.6% and 3.2% EF contained diet for 2 weeks prior to the administration of both direct-acting, mitomycin C (MCC, 1 mg/kg body weight, i.p) and indirect-acting, 7,12-dimethylbenz(a)anthracene (DMBA, 40 mg/kg body weight, i.g) clastogens. However, their anticlastogenic potential against both direct- and indirect-acting types of clastogens were found in mice[10]. However, the long-term consumption for 24-weeks in mice received EF should be avoided due to chronic toxicity. EF diet at 1.6% and 3.2% showed the adverse effects on some biochemical parameters (increased blood urea nitrogen) and histopathology (tubular degeneration of kidney) in mice after 24 weeks of treatment[11]. However, there was no report on the protein

expression profile in any organs of mice treated with EF. Therefore, shotgun proteomic approach was used to investigate the changes in mouse colon proteome after treated with EF for the safety concern of 15 weeks consumption.

World Health Organization (WHO) described that the incidence of colorectal cancer (CRC) is rapidly rising in various regions within countries such as China, Japan, Korea and Singapore. The increase in the number of new cases of CRC per year is witnessed in both men and women. However, not all countries in Asia witness the same degree of rise in incidence of CRC, for example, in East Asian countries, such as Indonesia, Thailand, Vietnam and India[12]. CRC in Thailand is the third most common type of cancer in men and the fifth most common type of cancer in women. The annual incidence per 100,000 persons in 2010-2012 of male and female were 14.4 and 11.2, respectively[13]. Early detection of CRC can therefore significantly reduce the mortality for this malignancy. However, current screening methods including faecal occult blood test (FOBT), sigmoidoscopy, colonoscopy and virtual computed tomography scanning either lack the required sensitivity and specificity or are costly and invasive[14]. Some biomarkers such as the circulating carcinoembryonic antigen (CEA) levels and tumor-associated gene mutations have only shown some prognostic or predictive value[15]. Therefore, there is an urgent need for developing new screening tests and identifying new biomarkers to diagnose, predict, and monitor the progress of CRC, and eventually find more efficient drug targets for this disease.

Materials and Methods

Eryngium foetidum Linn. leaves (EF) collection and preparation

The EF samples were collected from 4 different cultivation areas in Thailand. All samples were pooled and preparations were made according to method described by Promtes et al[9]. Briefly, edible part of the EF was washed with both tap water and deionized water, drained, and air-dried and then cut into small pieces. Then, the EF samples were quickly frozen, ground, sifted, and stored at -20˚C until used. Then aliquots of each EF sample were pooled and the AIN-76 diet was supplemented with 0.8% and 3.2% of freeze-dried EF before giving to mice.

Tissue specimens

The animal care and use was reviewed and approved by the National Cancer Institute, Thailand (AE.No.241/2011). Colon sample preparations were obtained from the study of Promtes et al[9]. The scheme for mice treatment is shown in Figure 1.

Sample preparation and GeLC-MS/MS analysis

Colon samples were used for proteomics analysis by using the combination of one-dimension SDS-PAGE and LC-MS/MS (GeLC-MS/MS)[16]. Briefly, proteins from colon tissues were extracted using 0.1% SDS (w/v), then equal amount of extracted proteins were fractionated on 12.5 % SDS-PAGE. The running condition was 20 mA/gel for approximately 90 minutes or until tracking dye reached the bottom of the gel. Electrophoresed gel was stained with silver staining[17]. To extract the proteins from gel, the in-gel digestion was performed according to Paemanee et al[16]. The LC-MS/MS analysis was performed as triplicated and described previously by Paemanee et al[16].

Protein identification and Gene ontology categories

Decyder MS Differntial Analysis software (GE Healthcare) was used to quantify the differentially expressed peptide in all samples[16]. The data obtained from Decyder MS was submitted to Mascot and searched against Musmusculus database for protein identification.

Gene ontology annotation and quantification of the change in protein analysis

The protein information was obtained from the online database especially, uniprot knowledge base database. All of the gene ontology information contained biological process. Proteins that showed a significantly altered expression level in the hierarchical clustering were identified by the multi experiment viewer (MEV) software[18]. To analyze the changes in the protein between control and treatment groups, the t-test significance of $p \leq 0.05$ and Pearson correlation were employed[16]. Other information including protein categorization and biological function were analyzed according to protein analysis through evolutionary relationships (Panther) protein classification[19].

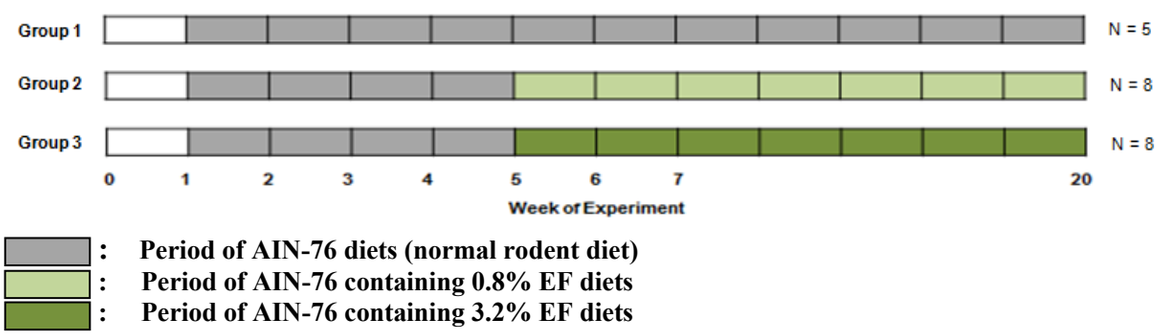

Figure 1 The experimental design of toxicity of EF in ICR mice (Promtes et al. 2013).

Results and Discussion

The differentially expressed protein in mouse colon treated with and without EF

Hierarchical clustering analysis of these proteins was performed to reveal the protein expression patterns. Comparing between mouse colon treated with and without EF, 1,856 differentially expressed proteins.

Analysis of peptide functions using gene ontology (GO)

The online PANTHER (Protein Analysis Through Evolutionary Relationships) was used to classify function of identified proteins. They were categorized into 14 groups according to their GO based functional annotation at the mouse genome annotation project (Fig. 2A-C).

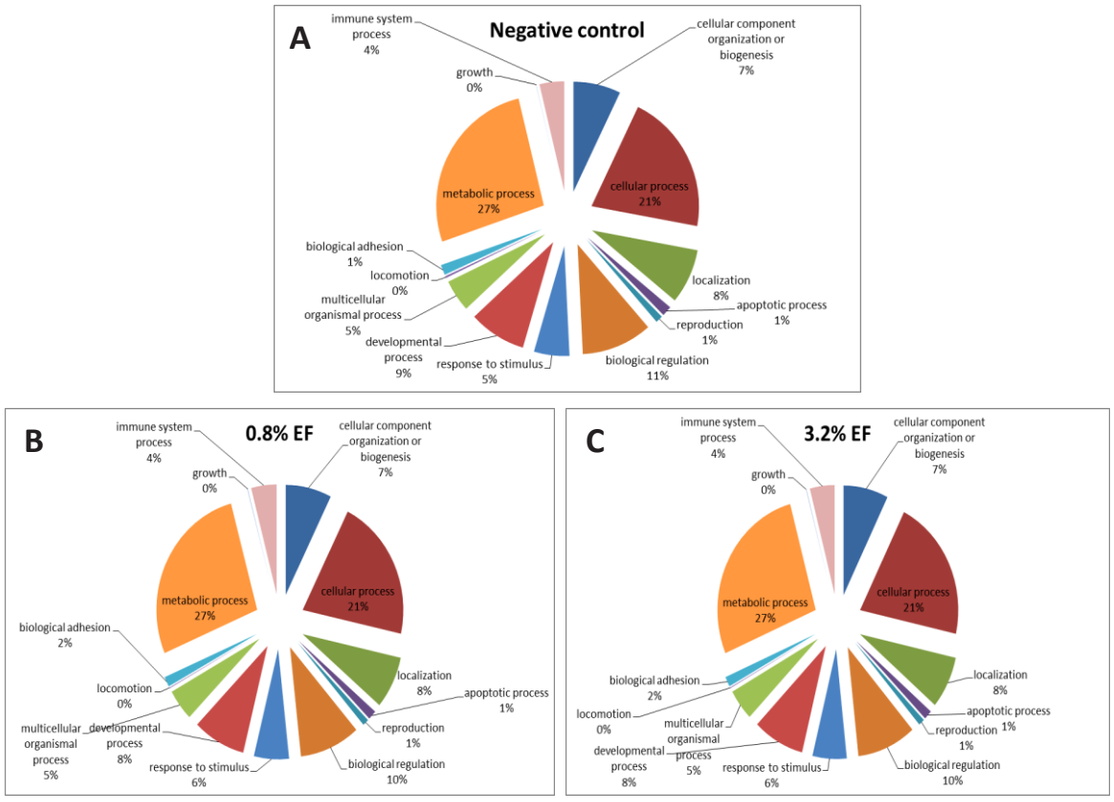

Figure 2 The functional distribution of 1856 proteins significantly differentially expressed in mouse colon after treated with and without 0.8% and 3.2% EF mixed AIN-76 diet for 15 weeks.

The data showed that the protein expression patterns of mouse colon in three sample groups (Group 1; control group, Group 2; 0.8% EF and Group 3; 3.2% EF in diet) were the same as shown in Figure 3, albeit at different levels of expression. This indicates that EF may affect the level of protein expression, but not for cellular integrity. In support, Promtes et al.[20] reported that EF does not induce colon carcinogenesis in mouse's colon after treatment with 0.8% and 3.2% EF for 15 weeks. However, a pathological study of mice treated with 1.6% and 3.2% EF in diet for 24 weeks displayed a mild grading on gut-associated lymphoid tissue (GALT) of small and large intestine[11]. Considerably, long-term treatment of EF more than 15 weeks should be considered for toxicity. The possibility of the adverse effect might be due to chronic consumption of high dietary fiber that may provoke inflammation in the large intestine[21].

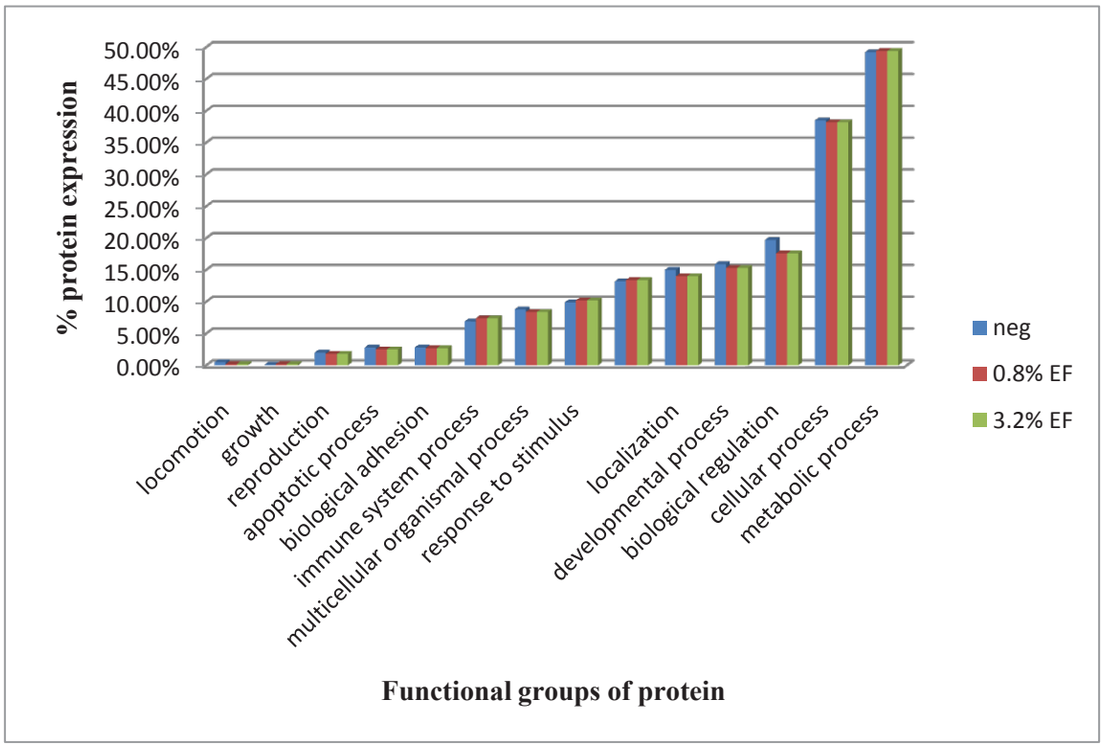

Figure 3 The proteins expression in mouse colon treated with and without 0.8% and 3.2% EF mixed AIN-76 diet for 15 weeks.

neg : Negative control group

0.8% EF : Group of mice received AIN-76 containing 0.8% EF diets

3.2% EF : Group of mice received AIN-76 containing 3.2% EF diets

The differences of protein expression between control and EF- treated mice were further examined. When compared with the control group, the EF-treated mice (0.8% and 3.2%) showed either increasing or decreasing of protein expression. The PANTHER showed that proteins involved in immune system process, response to stimulus and metabolic process were dramatically increased (Fig. 4 and Table 1). On the other hand, the proteins involved in biological regulation, localization and developmental process were reduced (Fig. 4 and Table 2).

It is possible that the induction of some proteins playing role in immune system process, response to stimulus and metabolic process might be due to the activation of biotransformation and antioxidant enzymes, such as phospholipid hydroperoxide glutathione peroxidase (PHGPx). It has been reported that PHGPx reduces product of lipid peroxidation and suppresses cell apoptosis. Moreover, PHGPX appears to activate a defense mechanism in order to reduce pro-inflammatory stimuli which is in line with the finding that NLRC5, a probable regulator of the NF-KB, was increased after EF treatment[22]. Conversely, the reduction of some proteins that function in biological regulation, localization and developmental process was observed. However, no pathological defect has been reported in the same group of mice treated with EF[20]. Thus, this suggested that reduction of these proteins might not contribute to typical cellular functions. Moreover, although the different levels of protein expression in mouse colon after EF treatment were not similar to control group, no alteration of proteins involved in colon's oncogenesis was detected.

The consumption of EF (0.8 and 3.2% in diet) did not alter protein expression pattern in mice within 15 weeks. Furthermore, the long-term use of EF at high dose should be avoided as the previous study illustrated its adverse effect on kidney function in mice[11].

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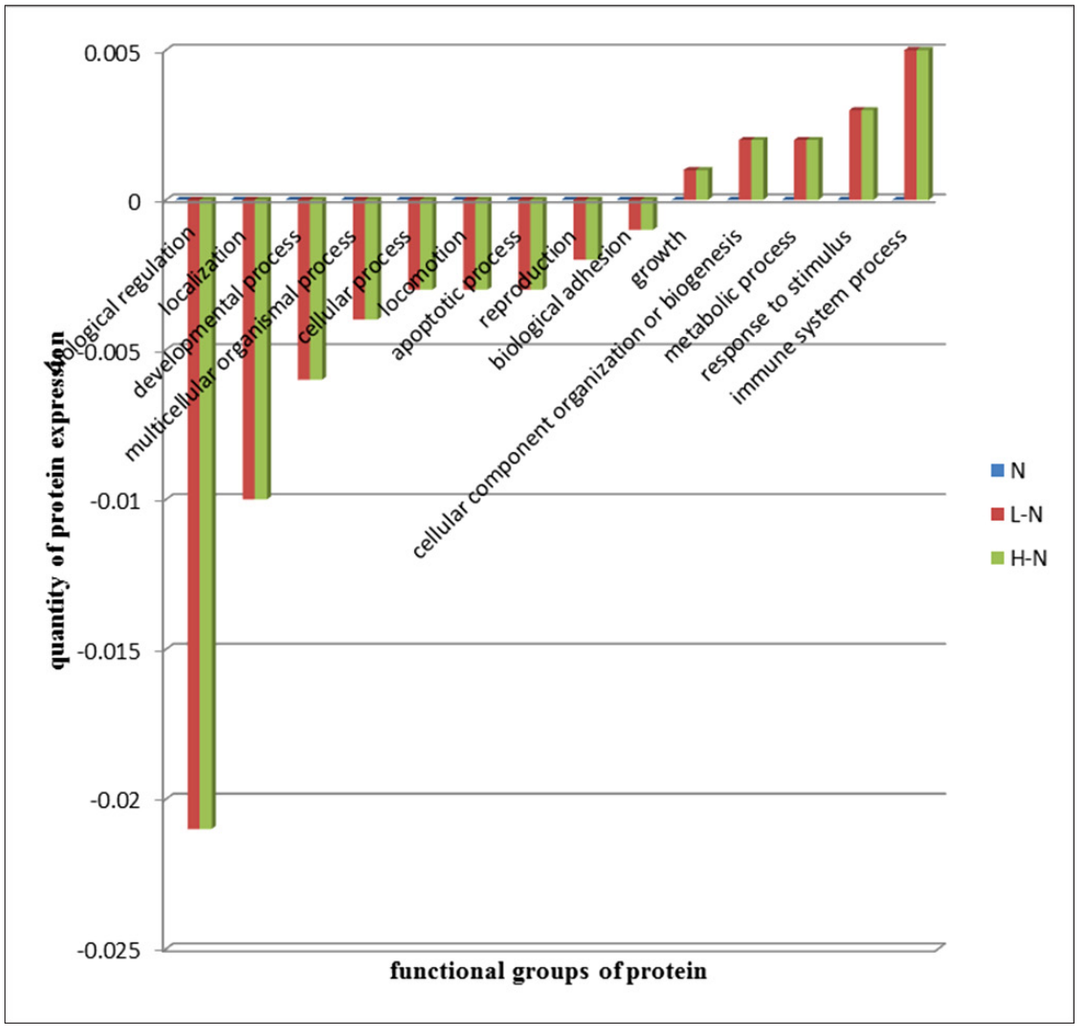

Figure 4 The change of proteins expression in mouse colon treated with and without 0.8% and 3.2% EF mixed AIN-76 diet for 15 weeks.

N : Negative control group

L-N : Quantity of protein in (0.8% EF group – negative control)

H-N : Quantity of protein in (3.2% EF group – negative control)

Table 1 Name and quantity of some upregulated proteins expression in mouse colon detected after mice treated with 0.8% and 3.2% EF mixed AIN-76 diet for 15 weeks.

Uniprot ID	Gene Name	Gene Symbol	PANTHER Protein Class	Quantity of Protein Expression (ng/200 µl)		
				Control	0.8%EF group	3.2%EF group
C3VPR6	Protein NLRC5	Nlrc5	transcription cofactor nucleic acid binding	**0**	**9.486**	**9.993**
O09039	SH2B adapter protein 3	Sh2b3	-	**0**	**8.336**	**8.498**
P63017	Heat shock cognate 71 kDa protein	Hspa8	Hsp70 family chaperone	**0**	**0**	**9.276**
P70375	Coagulation factor VII	F7	peptide hormone receptor serine protease protease inhibitor	**0**	**8.258**	**8.911**
Q60847	Collagen alpha-1(XII) chain	Col12a1	transporter receptor	**0**	**9.504**	**9.328**
Q8BQC3	Immunoglobulin superfamily DCC subclass member 3	Igdcc3	immunoglobulin receptor superfamily protein phosphatase immunoglobulin superfamily cell adhesion molecule	**0**	**0**	**10.382**
Q91XR9	Phospholipid hydroperoxide glutathione peroxidase, nuclear	Gpx4	peroxidase	**0**	**0**	**9.735**
Q9CWR0	Rho guanine nucleotide exchange factor 25	Arhgef25	signaling molecule guanyl-nucleotide exchange factor	**0**	**0**	**9.850**
Q9CYL5	Golgi-associated plant pathogenesis-related protein 1	Glipr2	defense/immunity protein	**0**	**0**	**8.123**
Q9D3W5	Leucine-rich repeat-containing protein 71	Lrrc71	transcription cofactor nucleic acid binding	**0**	**6.883**	**7.095**

Table 2 Name and quantity of some down regulated proteins expression in mouse colon detected after mice treated with 0.8% and 3.2% EF mixed AIN-76 diet for 15 weeks.

Uniprot ID	Gene Name	Gene Symbol	PANTHER Protein Class	Quantity of Protein Expression (ng/200 µl)		
				Control	0.8%EF group	3.2%EF group
A2AAJ9	Obscurin	Obscn	immunoglobulin receptor superfamily protein phosphatase immunoglobulin superfamily cell adhesion molecule	**6.024**	**6.626**	**0**
A2AD83	FERM domain-containing protein 7	Frmd7	guanyl-nucleotide exchange factor	**5.468**	**6.839**	**0**
G3X9R0	MCG68164	Cylc1	structural protein	**7.099**	**8.573**	**7.772**
O70624	Myocilin	Myoc	structural protein	**9.356**	**0**	**0**
O88487	Cytoplasmic dynein 1 intermediate chain 2	Dync1i2	microtubule family cytoskeletal protein	**4.154**	**4.385**	**0**
P04627	Serine/threonine-protein kinase A-Raf	Araf	non-receptor serine/threonine protein kinase non-receptor tyrosine protein kinase	**9.781**	**9.903**	**0**
P05213	Tubulin alpha-1B chain	Tuba1b	tubulin	**5.108**	**6.184**	**0**
P06332	T-cell surface glycoprotein CD4	Cd4	immunoglobulin receptor superfamily	**6.471**	**6.782**	**0**
P08553	Neurofilament medium polypeptide	Nefm	structural protein intermediate filament	**10.398**	**0**	**0**
P11679	Keratin, type II cytoskeletal 8	Krt8	structural protein intermediate filament	**10.587**	**10.421**	**9.739**
P13020	Gelsolin	Gsn	non-motor actin binding protein calcium-binding protein	**8.677**	**7.893**	**7.153**
P13412	Troponin I, fast skeletal muscle	Tnni2	non-motor actin binding protein	**6.808**	**7.387**	**6.919**
P28481	Collagen alpha-1(II) chain	Col2a1	transporter surfactant receptor extracellular matrix structural protein antibacterial response protein	**9.110**	**8.652**	**0**
Q8C310	Roundabout homolog 4	Robo4	immunoglobulin receptor superfamily protein phosphatase immunoglobulin superfamily cell adhesion molecule	**7.661**	**8.664**	**0**

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