Effects of Thuocnamtieuu: a Study in Mice Model of Induced Colorectal Tumors

Doan Minh Thuy[1], Ha Quang Phong[1], Nguyen Hoang Ngan[2], Pham Huyen Trang[1], Quach Thi Yen[1], Lu Doan Hoat Muoi[1], Tran Dinh Sinh[1], Pham Ngoc Tien[1], Dang Thi Lan Phuong[1]

[1] Vietnam University of Traditional Medicine

[2] Vietnam Military Medical University

SUMMARY

Objectives: *The present study was undertaken to evaluate the effects of Thuocnamtieuu in AOM/DSS-induced-colorectal tumors mice.*

Subject and method: *48 six-week-old Swiss mice were randomly divided into 06 groups. Two doses of Thuocnamtieuu were administered via intragastric gavage to Swiss mouse with AOM/DSS induced colorectal tumours. After 28 days treatment, all mice were sacrified. Assessment of the number and size of colorectal tumours, histopathological analysis were performed.*

Results: *The data have shown that Thuocnamtieuu has been effective in reducing significantly tumor volume (both benign and cancerous), but had not significantly decreased the number of tumors, reducing significantly the number of abnormal cells (cellular pleomorphism, Langerhang cells) in experimental colorectal cancer mice when compared to the control ones.*

Conclusions: *Thuocnamtieuu have the effect of reducing the colorectal tumors in a AOM/DSS-induced-colorectal tumors mice.*

Key words: *Colorectal tumour, Thuocnamtieuu, AOM/DSS.*

INTRODUCTION

Colorectal cancer is one of the most common malignancies in the world. According to New Global Cancer Data 2018, incidence of colorectal cancers accounted for 10.8% of all new diagnosed cases (1.8 million people) in which morbility was 9.2 % (881,000 people) [1]. In Vietnam, number of new cases in 2018, both sexes, all ages were 14 733 patients, accounting for 8.9%. Colorectal cancer ranks the fifth after liver cancer, lung cancer, stomach cancer and breast cancer [1].

Recently, surgery has been the main treatment method of colorectal cancer, the tendency was to combine surgery with radiotherapy, chemotherapy, immunotherapy [2]. During radiotherapy or chemotherapy, in addition to killing cancer cells, these therapies have severely damaged healthy cells, peripheral blood cells, including immune

Corresponding author: Dang Thi Lan Phuong
Phone number:(+84) 944143089
Email address: drlanphuong89@gmail.com

Received: 6/4/2023
Reviewed: 19/4/2023
Accepted: 30/5/2023

cells of the patients. Therefore, the patients have exhibited symptoms due to side effects of this therapy, such as fever, fatigue, anorexia, weightloss, insomnia, anemia, etc., which can make the patient worry.

In recent years, adopting traditional medicine methods in treatment cancer in general and colorectal cancer in particular has been a new trend to improve treatment effectiveness and quality of life, prolong life time for patients who have got colorectal cancer [3], [4], [5], [6], [7].

Thuocnamtieuu (TNTU) was fomulated with herbs which have effects of tonify qi and replenish blood, activate blood and resolve stasis, detoxify. Through clinical practice using this formular in patients with colorectal cancer after surgical, radiotherapy, chemotherapy, we found that it had the effects of recovering health, reducing pain, improving the quality of life. For evidence as a background for conducting in-depth studies as well as using it in clinical for treatment of colorectal cancer, the present study was undertaken to evaluate the effects of Thuocnamtieuu in a AOM/DSS-induced-colorectal tumors mice.

MATERIALS AND METHODS

Materials

TNTU formular consists of herbs: Radix Fallopiae multiflorae, Radix Astragali membranacei Fructus Aurantii, Rhizoma Curcumae longae, Radix Codonopsis javanicae, Hedyotis diffusa, Ganoderma.

All herbs met Vietnamese Pharmacopoeia IV standards.

TNTU was extracted with water to liquid form of 1:1 ratio (1mL equivalent to 1g of herbs) according to the routine procedure at the Pharmacy Department of Tue Tinh Hospital, Vietnam University of Traditional Medicine. Hanoi, Vietnam.

Instruments, Chemicals and reagents

Analytical scale 10-4, model CP224S (Sartorius - Germany)

A set of small animal surgical instruments and other experimental instruments.

Azoxymethane (AOM) and Dextran Sulfate Sodium (DSS) (Sigma, USA);

5-FU (5-Fluorouracil) tube 5ml, 50mg/1ml (Ebewe Arzneimittel GmbH, Austria).

Subjects:

48 Swiss mice, 6 weeks ages old, both male and female, meet the experimental standards, were obtained from the Laboratory Animal Center of National Military Medical University (Hanoi, Vietnam). Experimental animals, which were housed in groups of 4 per wire cage, were kept under standard laboratory conditions (12 hours of light, 12 hours of dark; 25°C) for 10 days to acclimatize to laboratory conditions. During acclimatization, standard mouse chow and water were available ad libitum.

Methods

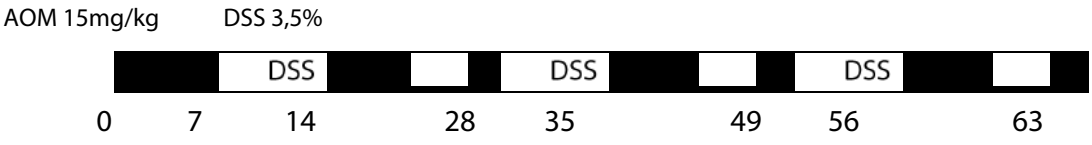
The study was designed as randomized controlled trials.

Research outcomes

General condition of mice, the number of tumors, the volumm of tumors, qualitative remarks of mice colorectal tumor histopathology.

Procedures

48 Swiss mice were separated into two groups: blank group (n = 8 ones) and 40 mice group were caused colorectal cancer with AOM/DSS for 63 consecutive days by Matthew A. Ciorba and et al [8].



Modeling colorectal cancer

On the 64th day, 48 Swiss mice were separated into six groups ($n = 8$ per ones):

The blank group (1);

The model (2): had been caused colorectal cancer, taken oral 0.9% NaCl (0.5ml/per day) and 0.9% NaCl intraperitoneal injection (0.4 ml / once /per week);

The 5FU (3): having been caused colorectal cancer, taken oral 0.9% NaCl (0.5ml/per day) and 5-FU intraperitoneal injection (50mg/kg/ once /per week);

The TNTU1 (4): having been caused colorectal cancer, taken oral TNTU 29.76g/kgBW and 0.9% NaCl intraperitoneal injection (0.4 ml /once /per week);

The TNTU2 (5): having been caused colorectal cancer, taken oral TNTU 59.52 g/kgBW and 0.9% NaCl intraperitoneal injection (0.4 ml /once /per week)

The (TNTU1 and 5FU) (6): having been caused colorectal cancer, taken oral TNTU 29.76g/kgBW and 5-FU intraperitoneal injection (50mg/kg/ once /per week)

The time the (5FU ,TNTU) groups took 5FU and/or TNTU was at the 64th day by the time mice had got colorectal cancer in 100% of the mice [Modelling of colorectal cancer was carried out on 96 swiss mice, on the 64th day, half of mice in all group (48 mice) were sacrified randomly to assess the colorectal cancer , the results showed that all 40 mice in the carcinogenic groups were suffered from colorectal cancer, the results were published in reference number 9 [9].

Two doses were extrapolated from human dose: 29,76g/kgBW/24h (Human dose multiply 12 times) and double dose 59,52g/kgBW/24h.

General condition of the mice were observed during the study.

After 4 weeks (28 days) taking TNTU, all mice were sacrificed, eviscerating colorectum of all in order to evaluate the number, the volume of tumors and histopathology.

The procedures of killing mice, expressing colorectal, assessing tumors, making histopathology specimens were carried out according to the guidance in the reference 7 [7]:

Assessing the number and size of tumours with a digital caliper.

Measurement of tumor size with caliper. Calculating tumor volume according to the following formula:

$V\ (mm^3) = \pi \times a^2 \times b/6$

In which, a is the smallest diameter of the tumor, b is the maximum diameter of the tumor.

Histological assessment

Three parts of the colon (proximal, mid, distal/rectum) were taken in uniform locations for each mouse, immobilized specimens in 4% paraformaldehyde, paraffin embedding, serially sectioned at 4 µm, and stained in hematoxylin and eosin for microscopic observation. Then perform assessment of histology [8].

Setting

The study was designed as randomized controlled trials, in which the setting was Department of Pharmacology, Department of

Anapathology of 103 Hospital, Vietnam Military Medical University, Hanoi, Vietnam.

Data analysis

All data analysis was carried out according to a pre-established analysis plan. Compared with anova test. Data was represented in the form mean(SD). The difference was statistically significant when $p<0.05$. The analysis was performed with the SPSS 16.0 software, free licence.

RESULTS

Effects of TNTU on general condition of mice

In blank group: all mice ate, activated normally, normal feces, dry anus. No mouse had abnormal expression;

In the control group, mice had manifestations of gastrointestinal disorders (diarrhea, mucus, hematochezia), do less activity, moodiness, puffiness, anorexia... recovered slightly after mice were no longer taken DSS. However, this situation persisted. In the last two weeks, the mice in the model showed more fatigue. At the 93th day, some mice showed a lot of fatigue, lying flat on the spot, did not eat and drink;

In (TNTU 1, TNTU2, TNTU1 and 5FU) groups, mice had manifestations of digestive disorders (diarrhea, mucus, hematochezia), reduced activity, moodiness, puffiness, poor appetite ... were significantly more improved than those in control group, no mice in these groups exhibited this appearances;

In the 5FU group, signs of decreased activity, moodiness, puffiness, poor appetite... improved compared to the control. However, gastrointestinal disorders symptoms (diarrhea, mucus, hematochezia) had not improved much compared to the control group.

Thus, TNTU had effect to improve the general condition of mice induced colorectal colorectalitis-associated cancer with AOM/DSS.

Effects of TNTU on colorectal tumors of mice

The blank group (n= 08): The colorectum of all mice was normal (without tumors or colorectalitis)

The mice groups induced colorectal cancer with AOM/DSS:

Macro examinations of mice colorectals were all found to have colorectal tumors.

Microscopic tests of mice colorectal tumors showed that there were all colorectal cancer.

Table 1. The effects of TNTU on the average number of colorectal tumors /mouse

Variables / Groups	Benign tumors		Cancer	
	mean (SD)	p	mean (SD)	p
Control (n=08)	1,13 (0,83)	> 0,05	1,88 (0,83)	> 0,05
5FU (n=08)	0,88 (0,64)		1,63 (0,92)	
TNTU 1 (n=08)	1,00 (0,76)		1,75 (0,89)	
TNTU 2 (n=08)	0,88 (0,83)		1,75 (0,71)	
TNTU 1+ 5FU (n=08)	1,00 (0,93)		1,63 (0,74)	

(SD: Standard deviation)

There were not statistically significant different in the average number of benign tumors and cancer masses between 5 FU, TNTU 1, TNTU 2, TNTU 1+ 5FU and the control ones.

Table 2. The effects of TNTU on the average volume of colorectal tumors (mm^3)

Variables	Benign tumors		Cancer	
Groups	mean (SD)	p	mean (SD)	p
The control (1) (n= 08)	1,25 (0,08)	$p_{-1} < 0,05$ $p_{3-4-5-2} > 0,05$	1,84 (0,16)	$p_{-1} < 0,05$ $p_{2-3-4-5} > 0,05$
5FU (2) (n= 08)	0,93 (0,06)		1,33 (0,15)	
TNTU 1 (3) (n= 08)	0,98 (0,05)		1,36 (0,18)	
TNTU 2 (4) (n= 08)	0,94 (0,08)		1,32 (0,11)	
TNTU 1+ 5FU (5) (n= 08)	0,90 (0,07)		1,31 (0,14)	

(SD: Standard deviation)

The average volume of tumors (including benign tumors and cancerous tumors) in TNTU groups and 5 FU groups were reduced statistically significant when compared to the model ones ($p < 0.05$). TNTU had effectiveness in reducing the volume of colorectal tumors in experimental mice.

When comparing between TNTU1, TNTU2 and 5FU group, there was no statistically significant difference in tumor volumes.

The mean volume of tumors of mice in TNTU 2 tends to be smaller than the 5FU, TNTU1. However, the difference was not statistically significant.

Table 3. Macroscopic anatomy of colorectal tumours in mice

Groups	Variables
The Control (n = 08)	2-4 tumors, 1-2mm^3 in size, tumours concentrated mainly in the distal colon and rectum. Colorectalitis injury (figure 1b).
5 FU (n = 08)	2-4 tumors with the size of them in which were smaller than in the control group, tumours also concentrated mainly in the distal colon and rectum. Cololitis injury was slighter than in the control ones (figure 1c).
TNTU 1 (n = 08)	2-4 tumors with size of them in which were smaller than in the control group, tumours also concentrated mainly in the distal colon and rectum. Colorectalitis injury was slighter than in the control ones (figure 1d).
TNTU 2 (n = 08)	2-4 tumors with size of them in which were smaller than in the control group, tumours also concentrated mainly in the dital colon and rectum. Colorectalitis injury was slighter than in the control ones (figure 1e).
TNTU 1+ 5FU (n = 08)	2-4 tumors with size of them (in which) were smaller than in the control group, tumours also concentrated mainly in the distal colon and rectum. Colorectalitis injury was slighter than in the control ones (figure 1f).

TNTU had effect to reduce the size of colorectal tumors equivalent to the 5FU.

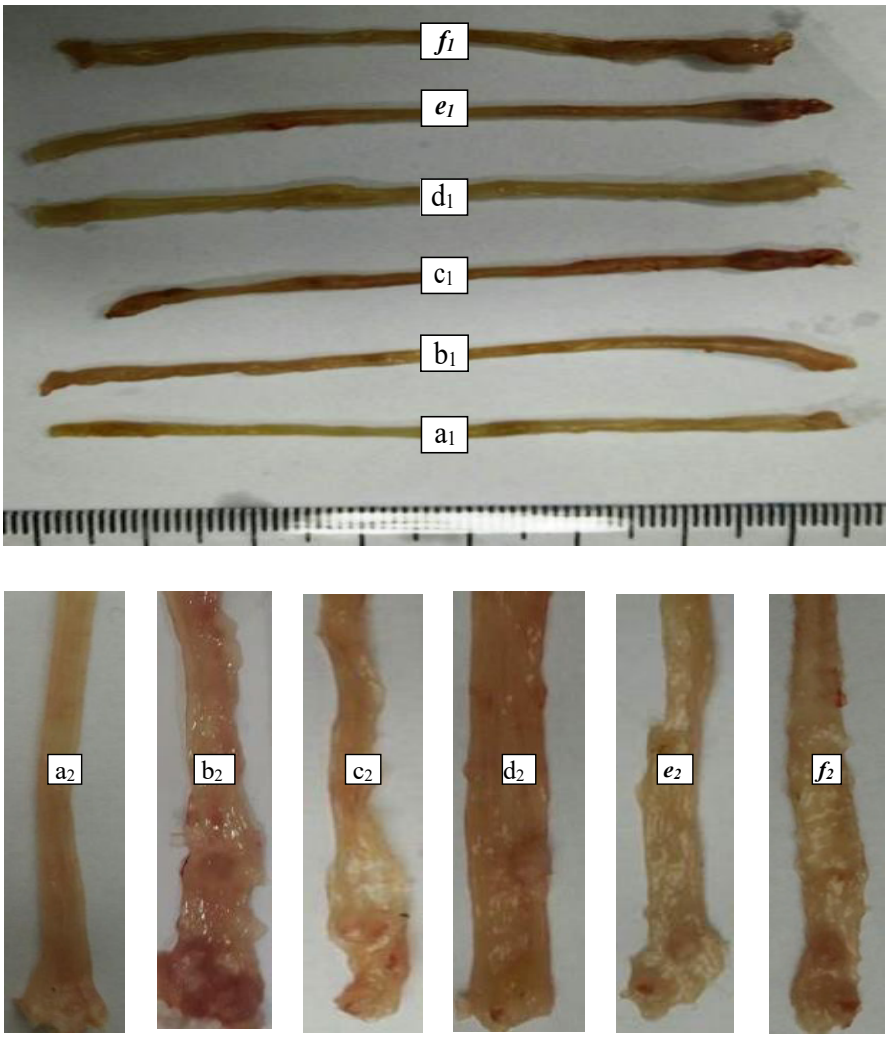


Figure 1. Colonrectal macroscopic anatomy (n = 8/ones)

a_1. Blank; b_1. Control; c_1. 5FU; d_1. TNTU1, e_1. TNTU2; f_1. TNTU1 and 5FU

(a_2; b_2; c_2; d_2; e_2;f_2 : the distal colon/retum of mice correspondingly)

Table 4 . Microscopic histopathology colorectal cancer in mice

Groups	Variables
The Control (n = 08)	Most cancer masses were larger in size, had numerous tumors of varying sizes; Cellular pleomorphism; The number of cells with Langerhans cells appeared; Manifestation of invasive mucosa (figure 2b).
5 FU (n = 08)	The size of tumours were usually smaller than compared to the control group; Cellular pleomorphism; the Langerhans cells appeared less than when compared those in the control group; no invasive ones (figure 2c).

TNTU 1 (n = 08)	The size of tumours were usually smaller than; the Langerhans cells appeared less than those in the control group; no invasive ones (figure 2d).
TNTU 2 (n = 08)	The size of the tumors were smaller; the Langerhans cells appeared less than those in above ones; no invasive ones (figure 2e).
TNTU 1 and 5FU (n = 08)	The size of the tumors were trully smaller; the Langerhans cells nucleus appeared less than those in above ones; no invasive ones (figure 2f).

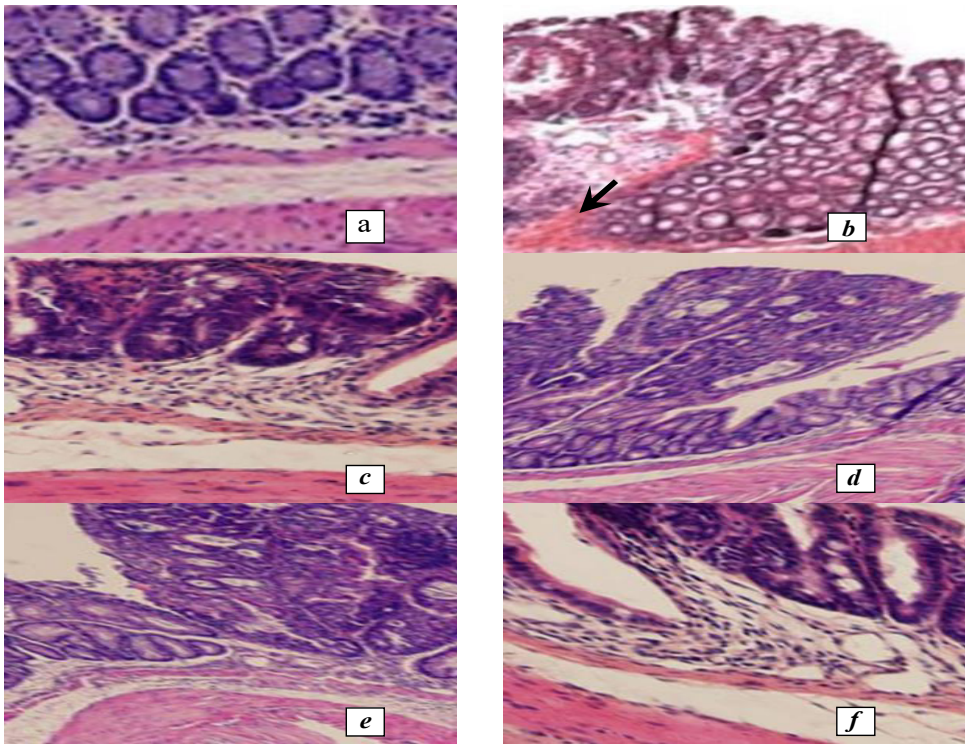


Figure 2. Colonrectal images (n = 8/ones) (HE x 400)

a. Blank (mouse 02, normal colorectum); b. Invasive mucosa and propria lamina ; c. d. e. f. Colonrectal carcinoma, no invasive mucosa; c. 5FU (mouse 05); d. TNTU1 (mouse 03), e. TNTU2 (mouse 06); f. TNTU1 and 5FU (mouse 08)

Thus, TNTU reduced the level of development of colorectal cancer, with magnifestation of the Langerhans cells appeared less than those in the control group; no mucosal invasive images. This result were equivalent between 5FU, TNTU1, TNTU2 and (TNTU1 + 5FU).

DISCUSSION

Colorectal cancer was known as one of the most common malignancies in the world. Many treatment methods of colorectal cancer such as surgery with radiotherapy, chemotherapy, immunotherapy are generally carried out by removing or killing cancer cells to reduce tumor size. In addition to killing cancer cells, these therapies have severely damaged healthy cells, peripheral blood cells, including immune

cells of the patients. Herbs is expected to have effect through improving the body's immunity, antioxidant, anti-inflammatory, limiting tumor growth, so on. The results of study showed that: suppling TNTU to mice induced colorectal cancer with AOM/DSS had reduced the level of development of colorectal cancer, with magnifestation of the Langerhans cells appeared less than those in the control group; no mucosal invasive images. This result were equivalent between 5FU, TNTU1, TNTU2 and (TNTU1 + 5FU).

Regarding to modeling colorectal cancer by AOM/DSS: In the control group, the data showed that all mice had showed digestive disorders, decreased activity, poor eating... Colorectal macroscopy anatomy showed tumours mainly in the distal colon and rectum, 2-4 tumour masses, 1-2 mm^3 in size (table 1, 2, 3, figure 1). Histological results showed cellular pleomorphism; the Langerhans cells appeared; invasive mucosa (table 4; figure 2). The data were also consistent with the ones of Matthew A. Ciorba and et al: numerous tumors of varying sizes with diameter mainly below 2mm, appearance of tumors in mouse colon, the higher tumor burden in the distal colon/rectum [8]. However, the number of tumors in our results were less than those of Matthew A. Ciorba et al. We hypothesized that it is possible experimental animals and experimental conditions were different, so lead to also different sensitivities. In addition, the mice were sacrificed on the 70th day in Matthew A. Ciorba model, while ours were on the 93th day, after stopping AOM / DSS for 28 days.

Treatment of mice with AOM/DSS effectively models colitis-associated cancer. Among them, DSS is an inflammatory substance. When mice were taken DSS, it caused chronic colorectalitis with symptoms of diarrhea, mucus, hematochezi,… [8]. The results showed that TNTU has the effects of improving the digestive disorder of mice significantly compared to the control group (Section: Effects of TNTU on general condition of mice).

5-FU is often indicated for the treatment of colorectal cancer, with high toxicity, it also may have contributed to aggravating digestive disorders in mice [10]. Therefore, in the 5FU group, this disorders had not improved much. In the group of TNTU combines 5-FU, those decreased significantly. Indicating that, TNTU had both worked to improve gastrointestinal disorders of mice and effectively reduced side effects of 5-FU. The combination between 5 - FU and TNTU should be further studied to evaluate the synergistic effects in treatment colorectal cancer, at the same time to find out the mechanism of these effects.

This effects of TNTU were probably due to herbs of formular that had the effects of tonify qi and replenish blood, activate blood and resolve stasis, enhancing immunity, anti-inflammatory [3][4][5][6][7]. However, there should be further research to find out this mechanisms.

The finding of reducing tumor volume (both benign and cancer), limiting tumor invasion in mice model of induced colorectal tumors, this results were not different between the control, TNTU1, TNTU2, TNTU 1 and 5FU. We hypothesized that it is possible because of the herbs of TNTU formular that had enhancing immunity, antioxidant [4][5][6][7], activating blood and eliminate stasis, detoxifying [3]. Therefore, the data have shown that TNTU had effective in improving the symptoms in a good way in mice of caused colitis-associated cancer, limiting development of cancer.

Regarding the number of tumors, the differences were not statistically significant when compared the TNTU groups to the control

ones. We hypothesized that, this limitations were due to the fact that the sample was not large enough, the time of taking TNTU were not enough to be effective (28 days). In addition, supplying of TNTU on mice that had suffered from colorectal cancer, so it was very difficult to reduce the number of tumors when compared to the model group. On the other hand, in our research, the number of tumors that were not many enough and the tumor growth were not fast, which may reduce the sensitivity of the model. Therefore, it is necessary to have further studies for a longer time, a larger sample size to clarify the mechanism and effects of TNTU.

CONCLUSIONS

These data indicate that TNTU had effects in improving digestive disorders; reducing significantly tumor volume; decreasing not significantly the number of tumors; reducing significantly the number of abnormal cells (cellular pleomorphism, Langerhang cells); no mucosal invasive in mice of inducing colorectalitis acsociated cancer that taken TNTU when compared to the control group. This result was equivalent between groups of TNTU1 and TNTU2 when compared to TNTU1 and 5FU group.

RECOMENDATIONS

Establishing the standards of TNTU and continuing to study to find out the mechanism of TNTU in treatment for colorectal cancer on larger sample size, longer time.

REFERENCES

1. International Agency for Research on Cancer. GLOBOCAN 2018, Online: https://www.uicc.org/news/new-global-cancer-data-globocan-2018.

2. International Agency for Research on Cancer. GLOBOCAN 2018, Online: http://gco.iarc.fr/today/data/factsheets/populations/704-viet-nam-fact-sheets.pdf.

3. Cai. Q., Lin. J., Wei et al. *Hedyotis diffusa* Willd Inhibits Colorectal Cancer Growth *in Vivo* via Inhibition of STAT3 Signaling Pathway. *International Journal of Molecular Sciences*, 2012, 13(5), pp. 6117–6128.

4. Eun Young Seo and Woo Kyoung Kim. Red Ginseng Extract Reduced Metastasis of Colon Cancer Cells *In Vitro* and *In Vivo, J Ginseng Res*; 2011, 35(3), pp. 315–324.

5. Hu. H., Ahn et al. *Ganoderma lucidum* extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. *Int. J. Cancer,* 2002, (102), pp. 250–253.

6. Na. Kun et al. Anticarcinogenic Effects of Water Extract of Sporoderm-Broken Spores of *Ganoderma Lucidum* on Colorectal Cancer *in Vitro* and *in Vivo*. *International Journal of Oncology,* 2017, 50.5, pp. 1541–1554.

7. Tseng A, Yang C-H, Chen C-H, et al. An *in vivo* molecular response analysis of colorectal cancer treated with Astragalus membranaceus extract. *Oncology Reports,* 2016, 35(2). pp. 659-668.

8. Ameet I. Thaker, Anisa Shaker, M. Suprada Rao, Matthew A. Ciorba. Modeling Colitis-Associated Cancer with Azoxymethane (AOM) and Dextran Sulfate Sodium (DSS). *Journal of Visualized Experiments,* 2012, 67, pp. 4100.

9. Phong. Ha Quang, Thuy Doan Minh, Ngan Nguyen Hoang. Perfecting colorectal cancer model on white mice. *Journal of Traditional Vietnamese Medicine and Pharmacy,* 2018, No. 02, pp.11-18.

10. Chun Seng Lee, Elizabeth J Ryan, Glen A Doherty. Gastro-intestinal toxicity of chemotherapeutics in colorectal cancer: The role of inflammation. *World J Gastroenterol*, 2014 April 14; 20 (14), pp. 3751-3761.