doi.org/10.36721/PJPS.2020.33.2.REG.621-625.1

Preparation of Uracil by bacteria isolated from *Morchella*

Shaobin Fu[1], Ye Zhang[1], Cailing Yang[1] and Qingfeng Meng[2*]
[1]Department of Pharmacy, Zunyi Medical University, Zunyi, China
[2]Department of Public Health, Zunyi Medical University, Zunyi, China

Abstract: *Morchella* is one of the most famous rare edible and medicinal fungi over the world. Highly nutritious and immature cultivation techniques led to the high price and the markets have remained tight. The pathogenic bacteria were serious in artificial cultivation of *Morchella* that affected the growth and yield of *Morchella*. Isolation of pathogenic bacteria and metabolites were investigated in order to improve the artificial cultivation technology. The isolated strain (YDJZ-01-01C) was identified by Gram staining and sequence of 16S rDNA. Structures of metabolites were confirmed based on NMR spectra and literatures. However, the main products were uracil and thymine that considered as important intermediate of anti-tumor 5-fluorouracil. Interestingly, a new synthetic pathway for preparation of uracil by microorganism was found except for chemical synthesis. The new preparation pathway provided mild, green, sustainable and environment friendly method to produce uracil that meets the needs of modern chemistry.

Keywords: Pathogenic bacteria, preparation pathway, uracil·*Morchella,* anti-tumor.

INTRODUCTION

Tumor is a serious public health problem that threatens human health and social economic development. The concern is that the morbidity and mortality are on the rise. Chemotherapy is considered as one of the necessary means to treat tumors (Naito et al., 2017; Mo et al., 2016). In recent decades, 5-Fluorouracil (5- FU) has always been the first choice for the treatment of esophageal cancer, gastric cancer, colon cancer, ovarian cancer, cervical cancer, lung cancer and breast cancer (Bwatanglan *et al*., 2016; Ledderhof et al., 2017; Kensara *et al*., 2016; Zou *et al*., 2016; Wu *et al*., 2016). Because of its prominent role and significant effect, it is still the preferred method for the treatment of malignant gastrointestinal tumor and it is also the basis of many standard chemotherapy regimens since the advent of 5-FU (Peng *et al*., 2016; Wei et al., 2016; Kitao *et al*., 2016; Koda *et al*., 2016). Therefore, fluorouracil has been applied in clinic for a long term since 1957 (Cui et al., 2017). High efficiency, low toxicity and high targeting fluorouracil drugs were eager for more research and clinic. Fluorouracil drugs, including fluorouracil, the precursor drugs and compound preparations were activated *in vivo* into the role of fluorouracil. The current listing drugs including 5-fluorouracil, tegafur, FUDR, Carmofur, Deoxifluridine and so on were mainly used for the treatment of gastrointestinal tumors and breast cancers. Fluorouracil-like drugs are in particularly more prominent in the treatment of medicine oncology with the wide application of oral and implantable dosage forms (Huang *et al*., 2017; Yamamoto *et al*., 2016; Kitamura *et al*., 2016).

Uracil is an important intermediate for the preparation of fluorouracil. In nature uracil commonly exists in marine organisms, particulate matters and dissolved seawater. It was reported that it can be produced by plants (Bassarello *et al*., 2004). However, the main method of production of uracil is organic synthesis. There were several literatures that reported the synthesis methods of uracil: (a) condensation reaction by β-dicarbonyl compounds or analogues and urea or thiourea to produce uracil (Mukeherjee *et al*., 2010); (b) β-dicarbonyl compounds or their analogues react with formamide and then react with ammonia to produce uracil (Harada *et al*., 1976) (c) dihydrouracil was obtained by cyclization of β-ureidopropanoic acid, uracil was produced after bromination and bromination of de-hydrogen bromide (Urjasz *et al*., 1999). The first method was adopted in industry to synthesize uracil. However, the activity of β-dicarbonyl compounds needed to be higher with urea as raw material that limited the scope of application. It was reported that uracil can be obtained by thiouracil via acidification after and then oxidation which showed obvious deficiency such as low yield, high cost and unfriendly environmental conditions. One-pot preparation of uracil by thiourea at normal temperature and pressure was also reported with 71% yield (Wang *et al*., 2014). Microbes as sources to produce active substances had long history. But there was no report about method of producing uracil by microorganisms. Authors found that *Morchella* grew slowly when inhibited by pathogenic microbes. Several strains including bacteria and fungi were isolated from pathogenic fruit body of *Morchella* in Guizhou, China. One strain of bacteria (Y DJZ-01-01C) identified as *Pseudomonas tolaasii* can obviously inhibit the growth of *Morchella* (fig. 1). Metabolites from the isolated strain were exploited in order to improve artificial cultivation. However, the two main substances were uracil and thymine. Herein, a bacterial strain isolated from *Morchella* provided a new synthesis pathway to produce the important intermediate uracil of anti-tumor 5- FU. The

**Corresponding author:* e-mail: qfmeng@126.com

preparation of natural products by microorganisms was one of the most popular methods with renewable resource.

MATERIALS AND METHODS

General experimental procedure

NMR spectra were recorded on an Agilent NMR Spectrometer operating at 400 MHz in DMSO-d_6. TLC analyses were carried out on pre-coated silica gel GF_{254} plates (0.25 mm thick, Qingdao Oceanic Chemicals, China). Column chromatography was carried out on silica gel (200-300 mesh, 300-400 mesh, Qingdao Oceanic Chemicals, China). Visualization of the TLC plates was performed by iodide spray reagent, 10% H_2SO_4 in 95% ethanol and phospho-molybdic acid. Three developing systems of TLC for product were carried out with chloroform: methanol, petroleum ether: ethyl acetate, petroleum ether: acetone respectively. General solvents and reagents were purchased from Chengdu kelong Chemical Industry Company, Chengdu, China.

Isolation and identification of Microorganisms

Microbial strains were isolated from pathogenic fruit body of *Morchella* from Tuanze Town in Guizhou, China. Segments by Micro-needle were placed on Petri dishes containing Water-agar medium in clean benche and were incubated at 25-28°C. Pure strains were obtained after several streak cultivations and then stored in Luria-Bertani (LB) slant medium at 4°C.

Gram stain and molecular sequencing methods were carried out to identify the active strain. 16S of ribosomal deoxyribonucleic acid (16S rRNA gene) was sequenced. The universal primers 27F (5′-AGAGTTTGAT CCTGGCTCAG-3′) and 1492R (5′- GGTTACCTTGTTA CGACTT-3′) were used to amplify the 16S from the DNA extracted (Zhao *et al*., 2011). The polymerase chain reaction (PCR) was performed with the following cycles: (1) 94°C for 5 min; (2) 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 40 s, and (3) 72°C for 10 min. Amplified products were sequenced by Beijing Bio-ulab Technology Co., LTD. Homology analysis was carried out on Blast software in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree by Neighbor-Joining (NJ) method was constructed by Mega 5.1.

Culture media

The media for isolation experiment was carried out in water agar medium (consisting of agar, 20g; distilled H_2O, 1000 mL) and on fermentation experiment was carried out in LB medium (consisting of peptone 10 g; yeast extract 5 g; sodium chloride 10 g; distilled H_2O, 1000 mL). The anti-bacterial activity was carried out on Mueller-Hinton Agar (MHA) medium (the power dissolved in ddH_2O was sterilization at high–pressure and then the plates were prepared at 50-60°C with 16 mL medium per plate).

Fermentation

Fermentations were carried out according to two-stage protocol. The purified cultures were grown in 200 mL Erlenmeyer flasks that contain 100mL LB media. The flasks were incubated at 37°C and 150 rpm on an incubator shaker. And then cultures were transferred into 1000 mL flasks containing 500 mL LB media and 2000 mL flasks containing 1000 mL LB media for another 5 days.

Isolation and identification of metabolites

After fermentation cultures were then extracted three times with EtOAc. The extracts were evaporated under reduced pressure and 1.3 g crude extract was obtained. The crude residue was first isolated by silica gel column chromatography with petroleum ether and ethyl acetate from 6:1 to 1:8. Fraction A and B were further purified with relatively high content by recrystalization and silica column chromatography. Three developing systems of TLC for product 1 were chloroform: methanol 6:1, petroleum ether: ethyl acetate 1:8, petroleum ether: acetone 1:1 respectively. Developing systems of TLC for product 2 were chloroform: methanol 3:1, petroleum ether: ethyl acetate 1:10, petroleum ether: acetone 1:1 respectively. And then a single point was presented by iodide, 10% H_2SO_4 in 95% ethanol and phospho-molybdic acid.

Antibacterial ability of compounds by Oxford cup method

Oxford cup method was carried out to screen the antibacterial ability of compounds. *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *C. albicans* ATCC 10213, *Proteus* species and *Brucella* species were selected and suspension was adjusted to 10^5 CFU/mL by DENSIMAT. The bacterial suspension was coated on MH agar plate by aseptic cotton buds upright. Oxford cups were placed on the plate coated by bacterial suspension. 5 mg Product 1 (thymine) and 7 mg product 2 (uracil) in 500 µL methanol were added into Oxford cup with methanol as the control. Plates were cultured at 37°C upright and the inhibition zones were measured in three replicates.

RESULTS

Isolation and identification of strain

Several strains including bacteria and fungi were isolated from pathogenic fruit body of *Morchella* in Guizhou and one bacteria strain (YDJZ-01-01C) was found strong activity of inhibiting the growth of *Morchella*. Gram stain and molecular sequencing methods were carried out to identify the active strain. Gram-negative bacterium was observed under a microscope (1000×) (fig. 2). Primer pair 27F and 1492R were used to amplify 16S rDNA sequence. Then the sequences of 16S rDNA were analysed on Blastn on NCBI web. The strain was confirmed as *Pseudomonas tolaasii* with 100% similarity. Phylogenetic

tree by Neighbor-Joining method was constructed by Mega 5.1(fig. 3). Number of branch indicates evolutionary rate. *Fictibacillus nanhaiensis* KJ372514 was selected as the outer group. Species belong to Escherichia clustered in one branch, and species belong to Pseudomonas clustered in another branch. Evolutionary rate of *P. tolaasii* (YGJZ-01-01C) and *P. tolaasii* - JQ782514 was the same.

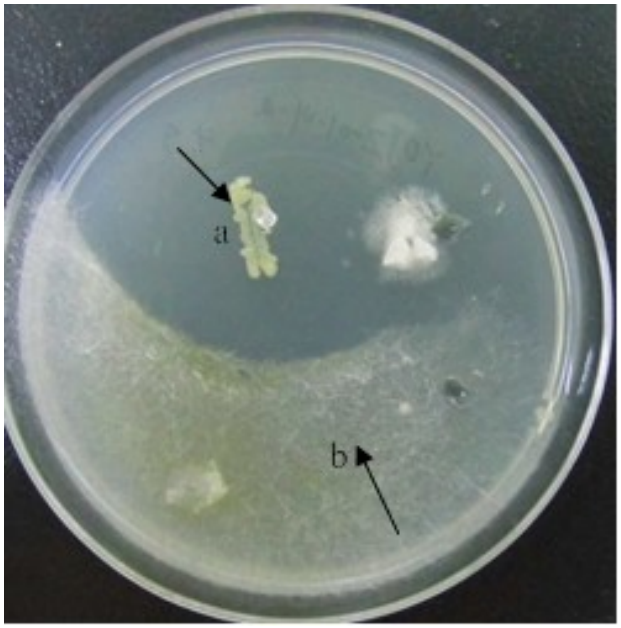

Fig. 1: Bacterial strain inhibited the growth of *Morchella,* a (YDJZ-01-01C), b (*Morchella*)

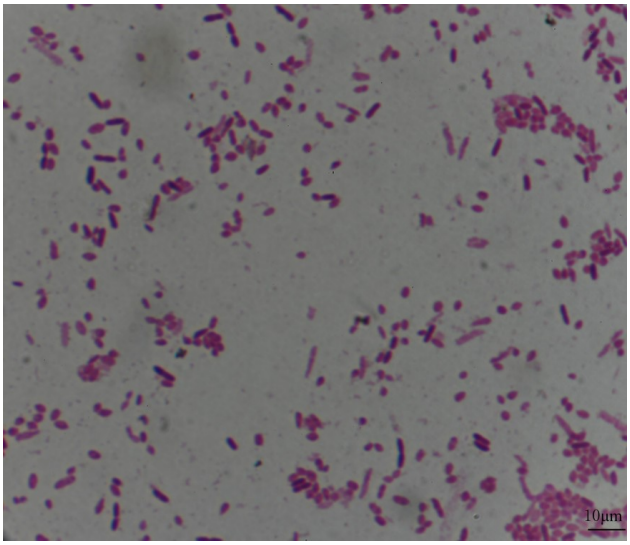

Fig. 2: Gram strain of *P. tolaasii* (YGJZ-01-01C)

Metabolites isolated from strain Pseudomonas tolaasii (YDJZ-01-01C)

Strain *P. tolaasii* (YDJZ-01-01C) with activity of inhibiting growth of *Morchella* was selected to screen new antibacterials. The main products were obtained by silica repeated gel column chromatography and recrystallization. Product 1: White powder solid. ^{1}H-NMR (DMSO-d_6) (fig. 4): 10.84(1H,s), 7.22(1H,s), 1.69(3H,5-CH3). ^{13}C-NMR (DMSO-d_6) (fig 5): 164.9(C-4), 151.5(C-2), 137.8(C-5), 107.7(C-5), 11.9(5-CH3). Compared with ^{1}H-NMR and ^{13}C-NMR, spectral data with those reported in the literature (Zou *et al*., 2004), its structure was confirmed as thymine.

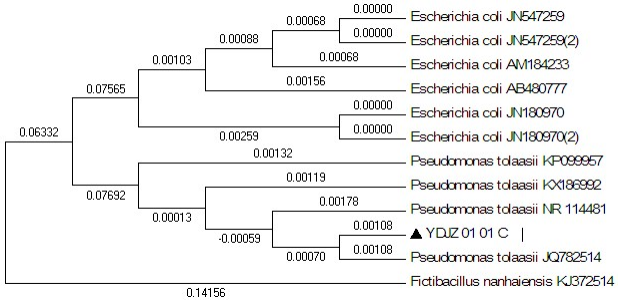

Fig. 3: Phylogenetic tree by Neighbor-Joining constructed using rDNA-16S sequences. ▲ indicated the isolated strain.

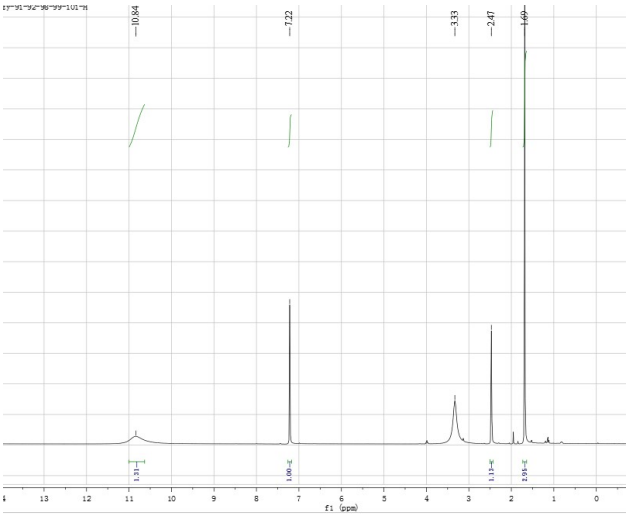

Fig. 4: ^{1}H-NMR spectraum of product 1 thymine

Product 2: White powder solid. ^{1}H-NMR (DMSO-d_6) (fig 6): 10.880(1H,s), 10.709(1H,s), 7.235(1H,d), 5.297 (1H,d). ^{13}C-NMR (DMSO-d_6) (fig. 7): 164.8(C-4), 152.0(C-2), 142.7(C-2), 100.7(C-6). Compared with ^{1}H-NMR and ^{13}C-NMR, spectral data with those reported in the literature (Zou *et al*., 2004), its structure was confirmed as uracil.

Antibacterial ability of compounds by Oxford cup method

Although strain *P. tolaasii* (YDJZ-01-01C) showed obvious inhibition of the growth of *Morchella*, metabolites 1 and 2 did not show significant activity against the pathogen *S. aureus* ATCC 25923, E. coli ATCC 25922, *C. albicans* ATCC 10213, *Proteus* species and *Brucella* species.

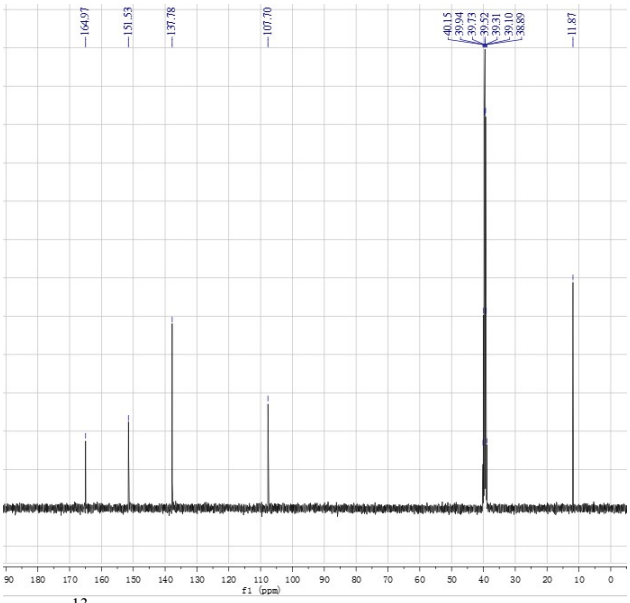

Fig 5. ^{13}C-NMR spectrum of product 1 thymine

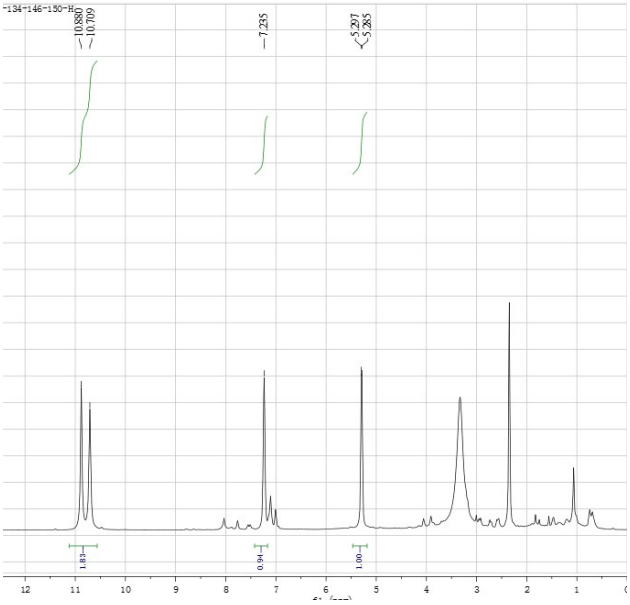

Fig. 6: ^{1}H-NMR spectrum of product 2 uracil

DISCUSSION

P. tolaasii was considered to be the main pathogen of several economically edible mushrooms. Brown blotch is also considered to be the cultivated mushrooms's main disease because of the important economic losses and the difficulty to control it. It was reported that the species can inhibit the growth of *Pleurotus ostreatus*, *Agaricus bisporus* and other mushrooms, and lead to brown spot disease. *P. tolaasii* is describes as the pathogen of some plants, indeed it caused disease on cauliflowers, tobacco and strawberry. Moreover, it was reported as saprophytic bacterium associated to pear, phyloplane and bean. Several compounds such as tolaasii I and tolaasii II and other five tolaasii A, B, C, D, E 5 can be produced by this species which might be important causes of disease symptoms in mushroom (Bassarello *et al*., 2004). Besides tolaasins, other compounds had been reported to be produced by *P. tolaasii* and considered as potential factors responsible for bacterial blotch symptoms. The bacterial volatile blends have been proved to interact with plants and fungi by inhibiting or stimulating their growth. *P. tolaasii* (YDJZ-01-01C) isolated from pathogenic fruit body of *Morchella* was proved to inhibit mycelia growth of *Morchella*. Metabolites of *P. tolaasii* (YDJZ-01-01C was investigated in order to establish the key compounds. The isolated compounds were thymine and uracil that has no anti-bacterial activity. However, the preparation of uracil that was considered as important intermediate of anti-tumor 5-fluorouracil by micoorganism seemed worthy of interest. The new preparation pathway provided mild, green, sustainable and environment friendly method to produce uracil that meets the needs of modern chemistry.

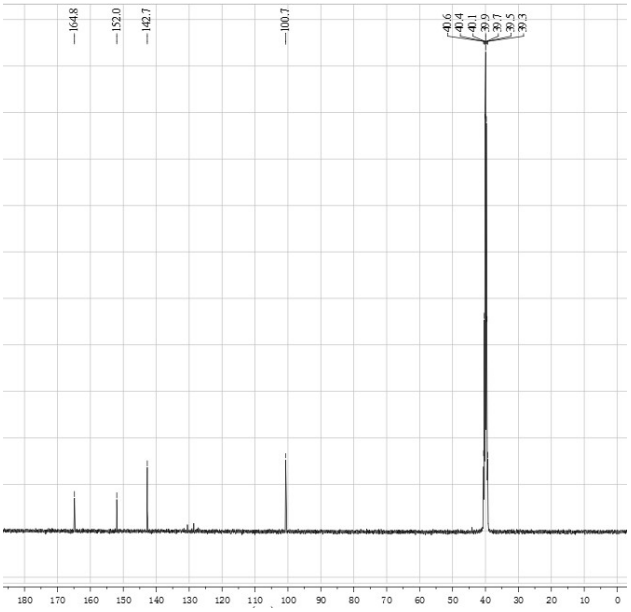

Fig. 7: ^{13}C-NMR spectrum of product 2 uracil

CONCLUSION

One pathogenic bacteria *P. tolaasii* (YDJ-01-01C) was isolated from edible fungi *Morchella*. The strain (YDJZ-01-01C) was identified by Gram staining and sequence of 16S rDNA. Two metabolites were obtained and identified as thymine and uracil. A new synthetic pathway for preparation of uracil by microorganism was found except for chemical synthesis.

ACKNOWLEDGEMENTS

This research was financially supported by The National Natural Sciences Foundation of China (No. 21462057),

Undergraduate innovation and entrepreneurship training program (201751105, 201710661037), Guizhou Science and Technology Department (QKHLH-2014-7555) and Program for Guizhou Key Laboratory of Microbial Resources & Drug Development (No.GZMRD-2014-003).

REFERENCES

Naito M, Yamamoto T, Shimamoto C and Miwa Y (2017). Retrospective Analysis of the Risk Factors for Grade IV Neutropenia in Oesophageal Cancer Patients Treated with a Docetaxel, Cisplatin, and 5-Fluorouracil Regimen. *Chemotherapy,* **62**(4): 215-224.

Mo WB, Su CH, Huang JY, Liu J, Chen ZH and Cheng KG (2016). Synthesis of acyl oleanolic acid-uracil conjugates and their anti-tumor activity. *Chem. Cent. J.,* **10**(1): 69-79.

Bwatanglan IB, Mohammad F, Yusof NA, Abdullah J, Alitheen NB, Hussein MZ, Abu N, Mohammed NE, Nordin N, Zamberi NR and Yeap SK (2016). *In vivo* tumor targeting and anti-tumor effects of 5-fluororacil loaded, folic acid targeted quantum dot system, *Journal of Colloid and Interface Science*, **480**(2): 146-158.

Ledderhof NJ, Caminiti MF, BradleyG, Bradley G and Lam DK (2017). Topical 5-Fluorouracil is a Novel Targeted Therapy for the Keratocystic Odontogenic Tumor, *J. Oral Maxillofac. Surg*., **75**(3): 514-524.

Kensara OA, Shemi AG, Mohamed AM, Refaat B, Idris S and Ahmad J (2016). Thymoquinone subdues tumor growth and potentiates the chemopreventive effect of 5-fluorouracil on the early stages of colorectal carcinogenesis in rats. *Drug Des. Devel. Ther.,* **10**(2): 2239-2253.

Zou XJ, Liang JY, Sun JY, Hu X, Lei L, Wu D and Liu L (2016), Allicin sensitizes hepatocellular cancer cells to anti-tumor activity of 5-fluorouracil through ROS-mediated mitochondrial pathway. *J. Pharmacol. Sci.*, **131**(4): 233-240.

Wu YH, Deng ZL and Wang HR, Ma WB, Zhou CX and Zhang SR (2016). Repeated cycles of 5-fluorouracil chemotherapy impaired anti-tumor functions of cytotoxic T cells in a CT26 tumor-bearing mouse model. *BMC Immunology,* **17**(1): 29-40.

Peng YC, Lu SD, Zhong JH, Xie ZB, You XM, Peng NF and Li LQ (2016). Combination of 5-fluorouracil and 2-morphilino-8-phenyl-4H-chromen-4-one may inhibit liver cancer stem cell activity. *Tumour Biol.,* **37**(8): 10943-510948.

Wei X , Duan W, Li Y, Zhang S, Xin XJ, Sun L, Gao M, Li Q and Wang D (2016). AT101 exerts a synergetic efficacy in gastric cancer patients with 5-FU based treatment through promoting apoptosis and autophagy. *Oncotarget,* **7**(23): 34430-34441.

Kitao H, Kiyonari S, Iimori M Niimi S, Kataoka Y, Akiyama S, Edahiro K, Nakanishi R, Tokunaga E, Saeki H, Oki E, Kanaji S, Kakeji Y and Maehara Y (2016). The molecular aspect of the antitumor effect of oxaliplatin in combination with 5-FU. *Gan To Kagaku Ryoho.,* **43**(6): 707-714.

Koda K, Miyauchi H, Kosugi C, Kaiho T, Takiguchi N, Kobayashi S, Maruyama T and Matsubara H (2016). Tumor 5-FU-related mRNA Expression and Efficacy of Oral Fluoropyrimidines in Adjuvant Chemotherapy of Colorectal Cancer, *Anticancer. Res*, **36**(10): 5325-5331.

Cui W, Fan WZ, Zhang Q, Wen J,Huang YH, Yang JY, Li JP and Wang Y (2017). Comparison of two transarterial chemoembolization regimens in patients with unresectable hepatocellular carcinoma: raltitrexed plus oxaliplatin versus 5-fluorouracil plus oxaliplatin, *Oncotarget*, **38**(5): 79165-79174.

Huang WY, Ho CL, Lee CC, Hsiao CW, Wu CC, Jao SW, Yang JF, Lo CH and Chen JH (2017). Oral tegafur-uracil as metronomic therapy following intravenous FOLFOX for stage III colon cancer, *PLoS One,* **12**(3): e0174280-90.

Yamamoto H, Murata K, Fukunaga M, Ohnishi T, Noura S, Miyake Y, Kato T, Ohtsuka M, Nakamura Y, Takemasa I, Mizushima T, Ikeda M, Ohue M, Sekimoto M, Nezu R, Matsuura N, Monden M, Doki Y and Mori M (2016). Micrometastasis volume in lymph nodes determines disease recurrence rate of stage ii colorectal cancer: A prospective multicenter trial. *Clin. Cancer Res.,* **22**(13): 3201-3208.

Kitamura Y, Kito S, Nakashima R, Tanaka K, Nagaoka K and Kitade Y (2016). Doxifluridine-conjugated 2-5A analog shows strong RNase L activation ability and tumor suppressive effect. *Bioorg. Med. Chem.,* **24**(16): 3870-3874.

Mukeherjee T, Zhang Y, Abdelwahed S, Ealick SE and Begley TP (2010). Catalysis of Flavoenzyme-mediated amide hydrolysis. J. Am. Che*m. Soc.,* **132**(16): 5550-5551.

Harada K and Suzuki S (1976). The new synthesis of uracil and 1,3-dimethyuracil, *Tetrahedron Lett.*, **17**(27): 2321-2326.

Urjasz W, Maciejewski A and Celewicz L (1999). Photochemical reaction of 5-fluropyrimidine bases with alcohols. *Tetrahedron Lett.,* **40**(16): 3243-3246.

Wang LM, Wang SY, Zhang ST, Yan DX and Jia YH (2014). Study on the synthesis of uracil as a pharmaceutical intermediate. *J. Liaoning Medical University,* **35**(6): 7-9.

Zou ZR, Yi YH, Yao XS, Du LJ, Zhou DZ and Zhang SY (2004). Studies on Chemical Constituents of Acaudina molpadioides Semper. *J. Nat. Med.,* **2**(6): 348-350.

Zhao LF, Xu YJ, Deng Z, Yang W and Wei G (2004). Identification and characterization of the endophytic plant growth prompter Bacillus Cereus strain mq23 isolated from *Sophora alopecuroides* root nodules. *Braz. J. Microbiol.,* **42**(2): 567-575.