

PRODIGIOSIN PURIFICATION FROM *SERRATIA MARCESCENS* M10 AND ITS ANTITUMOR ACTIVITIES

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SUMMARY

Prodigiosin (Pg) is a bright red pigment, which is produced by gram negative and gram positive bacteria including *Serratia marcescens*, *Hahella chejuensis*, *Vibrio psychroerythrus*, *Streptomyces coelicolor* and many other marine bacteria such as *Pseudomonas* sp., *Vibrio* sp. Pg induces apoptosis in different kinds of cancer cells with low toxicity on normal cells. In this study, we purified Pg from solid fermentation of *S. marcescens* M10 strain and assessed its anticancer activity in tumorized mice. The results showed that Pg was purified by running through a silicagel 60 column with a suitable solvent system of n-hexane:toluene at the rate of 1:1 (v/v) combined with toluene:ethyl acetate at the rate of 9:1 (v/v). TLC plate was used to test the presence of active substances with separation solvent n-hexane:ethyl acetate ratio of 1:1 (v/v). The purity of fractions tested by high performance liquid chromatography method showed as 98%. Purified fractions also showed promising anti-tumor activity in Lewis lung carcinoma induced tumors in BALB/c mice. The tumor volumes in Pg treated groups decreased by 34.18% after 28 days of administration.

Keywords: BALB/c mice, Lewis lung carcinoma, prodigiosin, *Serratia marcescens*

INTRODUCTION

Prodigiosin (Pg) is a member of family of natural red pigments which is called prodiginines. Pg is a secondary metabolite biosynthesized by various bacterial species in both gram-negative and gram-positive groups such as *S. marcescens*, *H. chejuensis*, *P. magnesorubra*, *V. psychroerythreus*... Among them, *S. marcescens* is the earliest known and most well-studied bacterial species used for Pg pigment production (Harned *et al.*, 1954). The red pigment Pg was studied by many scientists because of its great potential in medicine. Pg has been reported to have immune-suppressive, anticancer, antibacterial, antifungal, and

antioxidant activity (Ramina *et al.*, 2009; Priya *et al.*, 2013). In addition, it is thought to inhibit cell cycle and trigger apoptosis process (Montaner *et al.*, 2003). Furthermore, this is a substance with strong anti-cancer activity in human cancer cell lines and the cytotoxic activity of Pg was proved to affect tumor cells but not to normal cells (Pérez-Tomás *et al.*, 2003). In recent years, much attention has been paid to Pg due to its strong anti-cancer activity and mostly produced by bacteria. The studies on Pg production, bioactive tests and especially anticancer activity had made some progress. *In vivo* anticancer activities indicated that Pg significantly inhibited the growth of JEG3 and PC3 cells, and the inhibitory activity was dose

and time dependent (Dan Li *et al.*, 2018). Pg effectively inhibits tumor metastasis *in vitro* and *in vivo*. The action mechanisms of Pg are associated with the promotion of cell aggregation and the inhibition of various steps in cell invasive process, which include the inhibition of cell adhesion and mobility in a RhoA-dependent way and the suppression of MMP-2 ability (Jing Zhang *et al.*, 2005). In this study, we report an efficient purification of Pg by using toluene:ethyl acetate ratio 9:1 (v/v) and chloroform:ethyl acetate ratio 1:1 (v/v) solvents as well as its promising antitumor activity tested in benign induced BALB/c mice.

MATERIALS AND METHODS

Materials

Chemical, column chromatography silica gel and solvents (methanol, ethyl acetate, chloroform, acetone, toluene) used in this study were from Merck (Darmstadt, Germany) or other suppliers (China). Thin layer chromatography was performed on silica gel plates with 0.25 mm thick silica gel 60 F254 (Merck). Standard Pg was provided by Sigma Aldrich (St. Louis, MO, USA). *S. marcescens* M10 strain was isolated and identified by the members of the Enzyme Biotechnology Laboratory, Institute of Biotechnology (Vietnam Academy of Science and Technology - VAST) in previous studies. Albino BALB/c mice of 8-9 weeks of age were housed at the animal breeding area of the Institute of Biotechnology (VAST). All mice, which were healthy without any signs of diseases, regardless of breed, meeting the testing standards, were given standard food and free water. Metastatic Lewis Lung Carcinoma (LLC) cell line was used to induce cancer in mice (provided by Dr. Jeanette Maier, University of Milan, Italy).

Determination of Pg concentration by standard curve

Standard Pg was diluted at defined concentrations and measured OD value at a wavelength of 535 nm. This step was repeated

three times. The average value was taken to develop the standard curve of Pg, which was constructed based on the diluted Pg content and OD value at that dilution concentration. Pg was quantified by the standard curve $Y = 0.0562X - 0.0005$. The Pg content was determined by measuring OD value at 535 nm wavelength and was calculated according to the standard curve.

Extraction of Pg

The ethyl acetate:acetone solvent system at the ratio of 1:1, 2:1, 3:1 (v/v), respectively, were selected to extract the red pigment from the fermentation medium of *S. marcescens* M10 strain. The 1:1 (v/v) ratio of extraction solvent was kept in a shaker at 200 rpm for 3 hours at 28°C. The mixture was centrifuged at 4000 rpm at 4°C for 15 minutes. The solvent portion containing the red pigment was then extract by using a funnel.

Thin layer chromatography (TLC)

TLC was used to analyze and determine the number of different substances in extract components or separated fractions. TLC was performed on silica gel plates with 0.25 mm thick silica gel 60 F254 (Merck). In this study we selected the solvent system as chloroform:ethyl acetate as the mobile phase and the test ratio was 3:2; 1:1; 2:3 (v/v), respectively. The compounds were visualized by iodine staining. The ratio of the most suitable solvent separation system is used for further analysis.

Column chromatography

The crude extract was mixed with SiO₂ (20 g, Merck) at a ratio of 1:1 (w/w), then was put on a chromatography column (4x20 cm) by wet stuffing with methanol solvent. The extract in the column was eluted by 50 mL toluene:ethyl acetate (9v:1v) solvent system. Eluted fraction was analyzed by TLC. Clearly observed fractions were collected together, then put on chromatography column or dried for further studies.

Tumor suppression capacity of Pg using tumorized mice model

18 healthy BALB/c mice at 10-12 aged weeks, were tumorized by Lewis Lung Carcinoma (LLC) cell injection following the reported protocol (Do *et al.*, 2019). After 5 days of tumor induction, mice were randomly divided into three groups: (i) Pathological control group receiving water with a volume of 0.3 mL/mouse; (ii) Reference group receiving capecitabine at 200 mg/kg body weight (bw.) orally; (iii) Sample treatment group administering of Pg injection at 1.5 mg/kg bw. With the above experimental layout, we study the antitumor activities of the samples through inhibition of tumor growth. Tumor suppressive capacity of tested samples were compared with the pathological control group. The experimental mice were weighed and measured primary tumor sizes at the injection site every 7 days. The tumor volumes were calculated using the formula of Iigo *et al* (1991), Kimura *et al.* (2004) as $V = a \times b^2 / 2$ (where V: tumor volume; a: length of the tumor; b: diameter of tumor).

Data analysis

The data were processed in Excel, presented

in mean \pm SE. Student's t-test, F' test statistical algorithms, and one-way ANOVA analysis were used to check significant differences from the pathological control, with $p < 0.05$ is considered to be statistically significant difference.

RESULTS AND DISCUSSION

Optimization of the extraction solvent for Pg from M10 strain fermented broth

To select the most effective solvent system for Pg extraction from culture of *S. marcescens* M10, the crude Pg samples were run on a TLC with different solvent systems. In previous studies, several solvent systems have been used to extract and purify Pg. In study of Song *et al.* (2006), Pg was eluted with a mixture of n-hexane:ethyl acetate (2:1) (v/v), then was purified by TLC with chloroform:methanol:diethyl ether (6:2:2). Toluene:ethyl acetate (9:1) solvent system was used to eluted Pg from the silica gel 60 column (Zang *et al.*, 2014). This study focused on constructing several solvent systems to test the ability to separate Pg from other compounds in crude Pg extract using silica gel 60 for TLC.

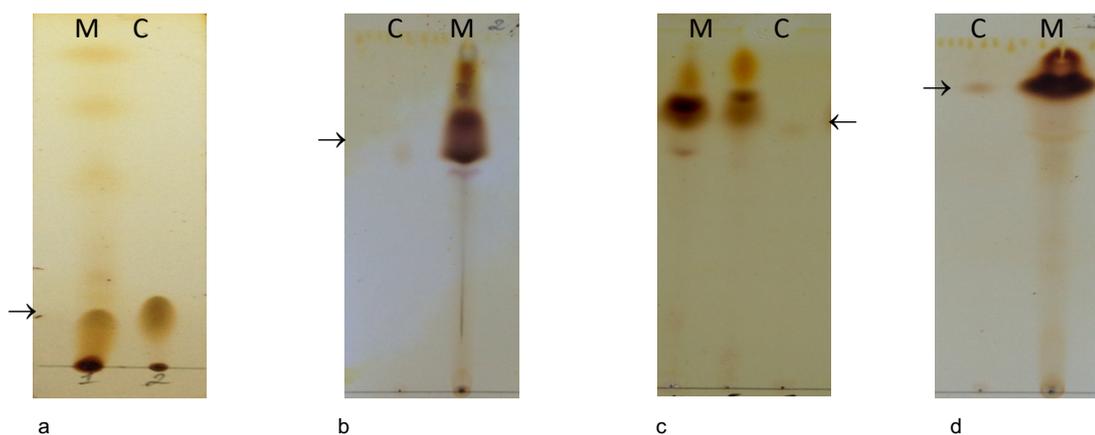


Figure 1. TLC analysis of Pg extract using different solvent systems. a, Toluene:ethyl acetate (9:1); b, Methanol:ethyl acetate:chloroform (6:3:1); c, Methanol:chloroform:acetone (4:3:2); d, Acid acetic:methanol:chloroform (65:30:5).

The results in Figure 1 indicated that using different solvent systems, the compounds were separated differently through silica gel column chromatography even they were from the same crude Pg extract. The toluene:ethyl acetate (9:1) solvent system was shown to be the best one to separate different compounds in the mixture of crude Pg extract (Figure 1a), especially the compounds with Rf lower than Pg. The remaining solvent systems such as methanol:ethyl acetate:chloroform (6:3:1), methanol:chloroform:acetone (4:3:2) and acetic acid:methanol:chloroform (65:30:5) did not

show clear and separated bands (Figures 1b-d). Our result that toluene:ethyl acetate was the most effective solvent for separating different compounds in crude Pg extract was consistent with the study of Zang *et al.* (2014).

Examining suitable ratio of toluene and ethyl acetate in solvent system for separating different compounds in crude Pg extract

Toluene:ethyl acetate solvent systems at different ratios were examined for TLC to determine the suitable ratio for separating Pg from other compounds in the crude Pg extract.

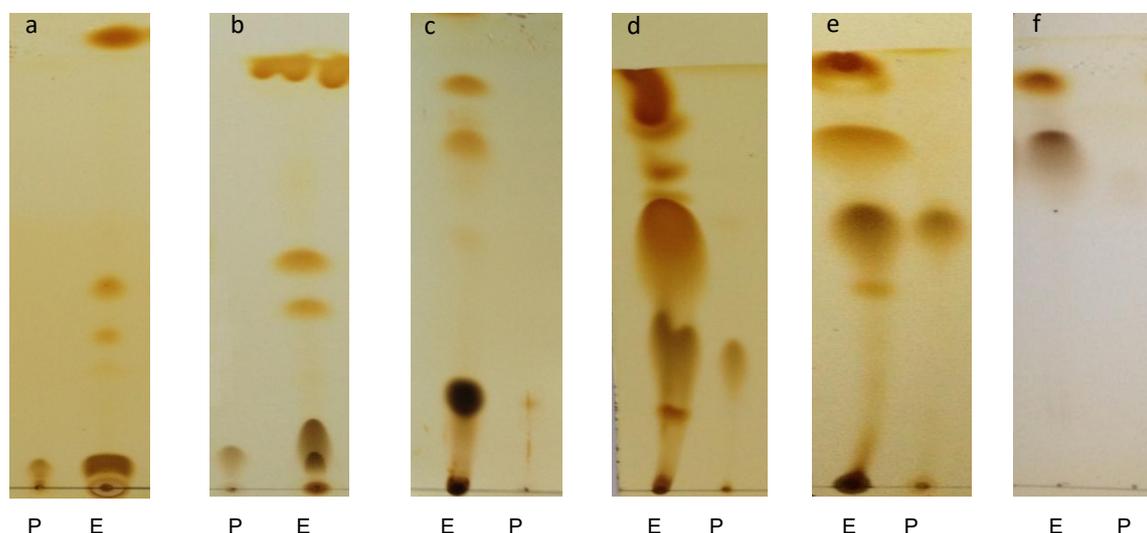


Figure 2. TLC with different ratio of toluene and ethyl acetate in the solvent system. a, 9:1; b, 4:1; c, 7:3; d, 3:2; e, 1:1; f, 1:4. E, Eluted fraction from column chromatography; P; Standard Pg.

The extraction of Pg from the crude Pg extract were conducted with TLC experiment on silica gel chromatography using toluene:ethyl acetate solvent systems at different ratios. The results showed that in higher concentrations of toluene, extraction of compounds with high Rf value was better than the extraction of Pg (Figure 2). In high concentration of toluene solvent (toluene:ethyl acetate 9:1 and 4:1), fractions from compounds with higher Rf value were easily isolated (Figure 2a, b). At the toluene:ethyl acetate ratios of 3:2, 1:1 và 1:4 (v/v)), the Rf value of Pg were 0.38, 0.54 and 0.72, respectively. That means the Rf values of Pg at these ratios were high. In lower concentration of

toluene solvent, isolation of Pg was slower from the crude Pg extract than the isolation of other components. Therefore, it is difficult to get pure Pg in low concentration of toluene (Figure 2d-f). In this experiment, Rf value of Pg in the toluene:ethyl acetate 9:1 and 4:1 were 0.125 and 0.19, respectively. Hence the toluene:ethyl acetate ratios of 9:1 and 4:1 could be the most suitable solvent ratio for purification of Pg from *S. marcescens* M10 culture extract using silica gel column chromatography.

Purification of Pg by silica gel column

20 g activated silica gel 60 in methanol was stuffed into a column (4x20 cm), then washed

and balanced with toluene:ethyl acetate (9:1) solvent system. 0.2 g crude Pg was dissolved in 2 - 3 mL of toluene:ethyl acetate (9:1) solvent, loaded onto the column and fractions were eluted by ethyl acetate:toluene solvent at a flow rate of 25 mL/hour. Purified Pg fractions were collected and checked by TLC and high-performance liquid chromatography (HPLC). The results showed that on TLC only a single band with the same Rf of standard Pg was observed (Figure

3a). As shown in the HPLC profile in Figure 3b, there was a single peak with retention time coinciding with the retention time of standard Pg.

The result clearly indicated that solvent system of toluene and ethyl acetate was the most effective solvent system for extraction of Pg from crude Pg extract. Pg could be purified with 98% purity by solvent system of toluene and ethyl acetate using silica gel column chromatography.

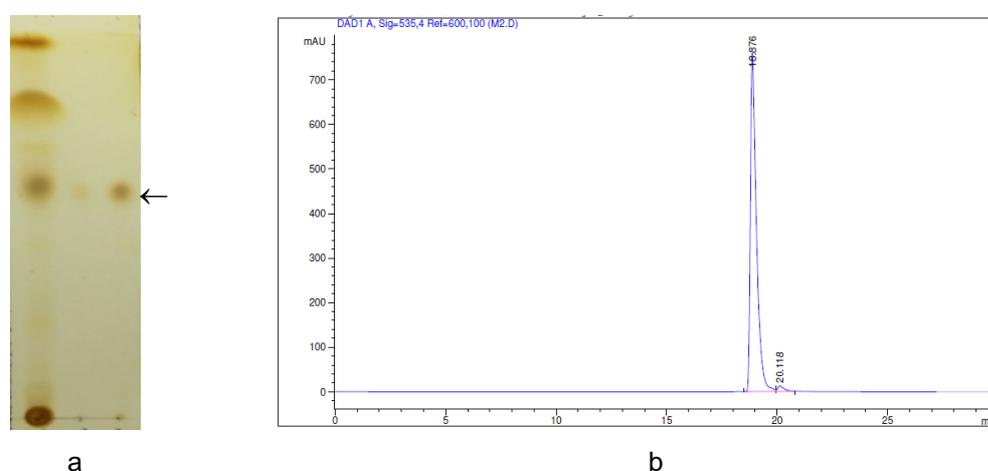


Figure 3. Pg purity after silica gel 60 column chromatography by TLC (a) and HPLC (b).

Assessment of antitumor activity in tumorized mice model

According to many recent researches, the LLC cell line is currently used as an experimental tumor-inducing factor in mice in many laboratories with high efficiency. In our experiments, using LLC cells (2×10^6 cells/mouse/time) to induce tumors in albino BALB/c mice with 95-100% efficiency (Do Thi Thao *et al.*, 2008). Therefore, the LLC cell line was used for experimental tumor induction in this experiment to evaluate the sample's ability to inhibit cancer growth *in vivo*.

Body weight of experimental mice

Average weight of experimental mice at five time-points (day 0, 7, 14, 21 and 28) were presented in Table 1.

The obtained results in Table 1 revealed that the average weight of experimental mice of the group that was Pg injected at the dose of 1.5 mg/kg and once in 2 days, was not different from that of control group ($p > 0.05$) at five time points (day 0, 7, 14, 21 and day 28) of the experiment. It was the same result for all mice which were capecitabine treated as reference group.

Tumor growth in experimental groups

Tumor growth is a very important indicator to assess the antitumor effect of the reagent. The research results on tumor growth are shown in Table 2.

The research results which were shown in Table 2 indicated that tumor volumes of the group that was Pg injected at the dose of 1.5 mg/kg/day, one time per 2 days at the day 14 and

day 21 were reduced in comparison with the control group. However, no statistical difference was found ($p > 0.05$). The same result was observed when comparing these two groups at the day 28, but the difference was statistically significant ($p < 0.05$). Nevertheless, there were two mice died quite early before the end of the experiment (died on day 17 and 19 from the start of injection of research sample). The dead mice

were also recorded in the pathological group of which were two mice and on day 22.

The tumor volume of mice in the reference group which were orally treated with capecitabine at the dose of 200 mg/kg/day, was decreased at all measured time points on day 7, 14, 21 and day 28. The statistical difference of the tumor volume in this group was founded ($p < 0.05$).

Table 1. Average weight of experimental mice (g/mouse).

Experimental Groups		Average weight of mice (g/mouse)				
		Day 0	Day 7	Day 14	Day 21	Day 28
Pathological control	Mean	35.79	34.86	34.86	38.17	41.13
	SE	1.15	1.35	1.35	2.13	2.27
Capecitabine at the dose of 200 mg/kg	Mean	35.08	34.02	34.53	35.08	37.03
	SE	1.16	1.51	1.97	2.31	2.08
Sample at the dose of 1.5 mg/kg/day, 1 time/2days	Mean	35.40	35.24	34.30	34.50	38.33
	SE	2.32	2.24	2.41	3.35	3.81

Note: * $p < 0.05$ in comparison with the negative control group.

Table 2. The growth of the tumor (mm^3).

Experimental Groups		Tumor volumn (mm^3)				
		Day 0	Day 7	Day 14	Day 21	Day 28
Pathological control	Mean	630.00	2654.78	5455.33	8807.25	12040.31
	SE	101.02	217.35	659.74	1241.90	1059.19
Capecitabine	Mean	608.29	1806.45*	3034.39*	4789.54*	7149.05*
	SE	79.70	313.65	432.70	751.65	1350.02
Pg	Mean	606.67	2207.21	3994.70	6284.93	7925.37*
	SE	137.71	364.21	945.61	1622.16	1846.89

Note: * $p < 0.05$ in comparison with the negative control group.

The ability to inhibit tumor growth of the experimental samples

As observed in the table 3, results revealed

that the tumor volume of Pg which were injected at the dose of 1.5 mg/kg/day, one time per two days, was decreased 34.18% after 28 days of

experiment in comparison to that of the control group. These results had statistical significance in one-way ANOVA analysis ($p < 0.05$). The tumor volume of mice in the reference group which was used capecitabine at the dose of 200 mg/kg/day, was remarkably decreased compared to the control group at day 7, 14, 21 and day 28. The statistical difference in this group was founded ($p < 0.05$).

Again, as showed in the table 4, the average of tumor weight of all mice which were administered with Pg was decreased to 8.32 g/mouse, compared with 12.73 g/mouse of the control group. However, the difference was not significant statistical ($p > 0.05$) since the size of the experiment was small ($n=6$).

Hematological and several biochemical indicators at the last time point of the experiment

The obtained results in table 5 showed that the white blood cells of mice which were injected at the dose of 1.5 mg/kg/day, one time per two days, was decreased after 28 days of experiment in comparison to the white blood cells of mice in

control group. These results had statistical significance in one direction ($p < 0.05$). There was a decrease in number of red blood cells, hemoglobin but no statistically significant was found ($p > 0.05$).

The number of white blood cells of mice which were given capecitabine at the dose of 200 mg/kg/day in 28 days was decreased comparing to those of mice in control group. These differences were statistically significant ($p < 0.05$). The remaining hematological indicators did not differ compared with the control group.

The AST, ALT and creatinine indicator of mice which were injected at the dose of 1.5 mg/kg/day, one time per two days, was found to not significant decrease after 28 days of experiment compared to those indicators of mice in the control group (Table 6).

The AST, ALT and creatinine indicator of mice which were taken capecitabine at the dose of 200 mg/kg/day in 28 days was not decreased comparing to those of mice in control group.

Table 3. Percentage of tumor suppression at five time points.

Experimental Groups	% on tumor inhibition				
	Day 0	Day 7	Day 14	Day 21	Day 28
Pathological control	0	0	0	0	0
Capecitabine	3.58	31.95*	44.38*	45.62*	40.62*
Pg	3.20	16.86	26.77	28.64	34.18*

Note: * $P < 0.05$ in comparison with the negative control group.

Table 4. Tumor weight at the day 28 of the experiment.

Experimental Groups	Tumor weight at the day 28 of the experiment (g/mouse)
Control	12.73 ± 3.09
Capecitabine	7.70* ± 2.98
Pg	8.32 ± 4.43

Note: * $p < 0.05$ in comparison with the negative control group.

Table 5. Hematological indicators at the last time point of the experiment.

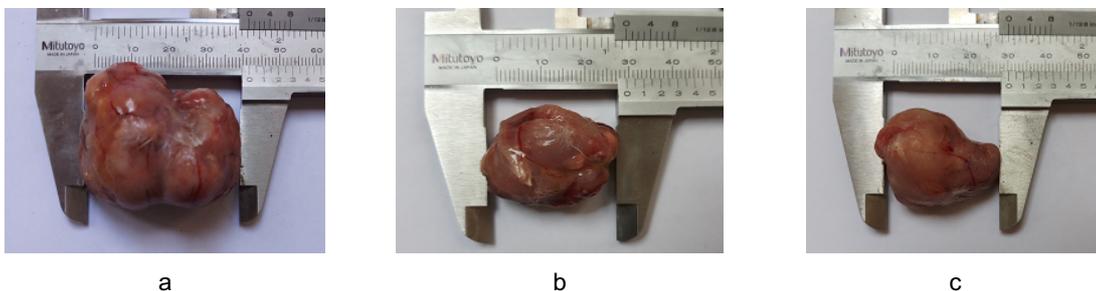
Indicators	Control		Capecitabine		Experimental samples	
	Mean	SE	Mean	SE	Mean	SE
White blood cell ($10^9/L$)	44.65	5.84	10.40*	2.55	16.61*	3.88
Red blood cell ($10^{12}/L$)	6.39	0.19	7.16	0.37	5.94	0.51
Hemoglobin (g/L)	92.33	2.60	105.00	12.00	87.75	6.28
HCT (L/L)	0.33	0.01	0.38	0.03	0.33	0.03
MCV (fL)	51.97	0.85	53.15	2.05	56.83	3.35
MCH (pg)	14.50	0.20	14.65	0.85	14.88	0.71
MCHC (g/L)	279.00	1.73	275.00	6.00	262.50	6.30
CHCM	259.67	3.18	261.00	2.00	255.25	5.07
CH	13.47	0.32	13.85	0.65	14.48	0.83
RDW (%)	15.97	0.56	16.85	0.95	19.43	1.51
HDW (fL)	20.07	1.05	20.45	2.05	22.40	1.40
Platelet ($10^9/L$)	574.00	16.07	566.50	5.50	567.25	9.44
MPV (fL)	4.87	2.14	6.65	0.35	7.15	1.94

Note: * $p < 0.05$ in comparison with the negative control group.

Table 6. Biochemical indicators at the last time point of the experiment.

Indicators	Control		Capecitabine		Experimental samples	
	Mean	SE	Mean	SE	Mean	SE
AST (U/l)	748.67	39.49	776.75	56.25	790.55	82.20
ALT (U/l)	50.93	5.39	35.75	5.15	42.00	1.98
Creatinin (micromol/L)	29.40	0.92	27.50	0.80	29.08	2.11

Note: * $p < 0,05$

**Figure 4.** Tumors after the experiment. a, Control; b, Capecitabine at the dose of 200 mg/kg; c, Sample at the dose of 1.5 mg/kg/day, 1 time/2 days.

DISCUSSION

The red pigment Pg is of great interest to many scientists and researchers because of its great potential in medicine. Pg has immunosuppressive, anticancer, antibacterial, antifungal, and antioxidant activity (Ramina *et al.*, 2009; Priya *et al.*, 2013). Pg has been shown to inhibit the cell cycle and trigger apoptosis (Montaner *et al.*, 2003). However, the anti-tumor activity in a mouse model (LLC) of Pg has not been reported in any previous study. In this study, we have created a process for extraction and purification of a large amount of Pg (0.6 g crude extracted Pg). An extraction solvent system of ethyl acetate:acetone of 2:1 (v/v) was shown being suitable to extract Pg from the bacterial cells. The suitable solvent system in plate TLC for the separation of compounds in crude Pg was chloroform:ethyl acetate at the ratio of 1:1 (Figure 3). The anti-tumor activity of the Pg has not been reported in any previous study. *In vivo* trial showed that our purified Pg active ingredient significantly reduced tumor volume (Tables 3 and 5) and number of leukocytes compared to the control. However, the rat's body weight, tumor weight and the AST, ALT, and creatinine parameters differed but not statistically significant compared to the control (Table 1, 2, 4 and 6). This result was similar to some other studies. *In vivo* trials of the active ingredient TAT2 extracted from *A. tonkinensis* leaves have been shown to significantly inhibit tumor growth at 100 and 200 mg/kg body weight (Thuy TT *et al.*, 2016). The AGN ethanol extract was tested against the growth of mouse Lewis Lung Carcinoma (LLC) allograft syngenic mice or human PC-3 and DU145 prostate cancer xenograft in immunodeficient mice. The pharmacokinetics of decursin and DA were determined. The AGN extract significantly inhibited LLC allograft growth (30 mg/kg) and PC-3 and DU145 xenograft growth (100 mg/kg) without affecting the body weight of the host mice (Hyo Jeong Lee *et al.*, 2009). The compound heyneanol A was extracted from Rupr *Vitis amurensis*. (Vitaceae) dose-dependently decreased tumor growth without any adverse

effect on body weight and seemed more potent than RES. The tumor inhibitory effects were accompanied by a marked increase in tumor cell apoptosis detected by cleaved caspase-3 and TUNEL assays and decreased tumor cell proliferation index and tumor microvessel density, supporting the involvement of apoptotic and anti-angiogenic activities in the anticancer effects (Eun -Ok Lee *et al.*, 2006). The results of this study showed that our purified Pg is a promising candidate for anti-cancer drug.

CONCLUSION

We have extracted Pg from solid fermentation media of *S. marcescens* M10 strain with a solvent system of toluene:ethyl acetate at the ratio of 9: 1 (v/v). Suitable separative solvent system in TLC was shown to be chloroform:ethyl acetate of 1:1. Extracted crude Pg was successfully purified by a silica gel 60 column chromatography being as a single band on TLC and a single peak of 98% purity with a Rf equal to the standard Pg on HPLC. The anti-tumor activity test with the LLC cell line in mice showed that after 28 days, the tumor volume decreased by 34.18% and the number of leukocytes decreased compared to the control ($p < 0.05$).

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TINH SẠCH VÀ ĐÁNH GIÁ HOẠT TÍNH KHÁNG U CỦA HOẠT CHẤT PRODIGIOSIN TỪ CHỦNG *SERRATIA MARCESCENS* M10

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TÓM TẮT

Prodigiosin (Pg) là một sắc tố màu đỏ tươi, được tạo ra bởi các vi khuẩn gram âm và gram dương

bao gồm *Serratia marcescens*, *Hahella chejuensis*, *Vibrio psychroerythrus*, *Streptomyces coelicolor* và nhiều vi khuẩn biển khác như *Pseudomonas sp.*, *Vibrio sp.* Pg được chứng minh gây ra quá trình tự chết (apoptosis) ở các loại tế bào ung thư khác nhau nhưng ít ảnh hưởng tới các tế bào thường. Trong nghiên cứu này, chúng tôi đã tinh sạch Pg từ chủng *S. marcescens* M10 và đánh giá hoạt tính chống ung thư trên chuột mang khối u. Kết quả cho thấy, Pg được tinh sạch bằng cách chạy qua cột silicagel 60 với hệ dung môi thích hợp là n-hexan:toluene tỷ lệ 1:1 (v/v) kết hợp với toluene:ethyl acetate tỷ lệ 9:1 (v/v) với Rf 0,125. Sự có mặt của hoạt chất prodigiosin sau khi tinh sạch được kiểm tra bằng sắc ký bản mỏng với hệ dung môi phân tách thích hợp là n-hexane:ethyl acetate tỷ lệ 1:1 (v/v). Pg sau khi tinh sạch được kiểm tra bằng phương pháp sắc ký lỏng cao áp với độ tinh khiết đạt 98%. Kết quả đánh giá hoạt tính kháng u của Pg tinh sạch với dòng tế bào ung thư Lewis lung carcinoma trên mô hình chuột BALB/c cho thấy Pg đã ức chế 34,18% thể tích khối u sau 28 ngày.

Từ khóa: BALB/c mice, Lewis lung carcinoma, prodigiosin, *Serratia marcescens*