

Study on the Preparation, Quantification and Antitumor Activity of Total Phenolic Acids in *Phyllanthus emblica*.

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Abstract

The optimal conditions for preparation of total phenolic acids in *Phyllanthus emblica*, and to study their inhibitory effect on human hepatoma cells have been investigated. The conditions for extraction of phenolic acid constituents in *Phyllanthus emblica* were investigated, and single factor and orthogonal tests were performed on conditions such as extraction solvent, extraction temperature, extraction time and solid-liquid ratio, respectively. To determine the inhibition ratio of drugs on cancer cells in different concentrations and periods by MTT method, and make quantitative determination for apoptosis by Flow cytometry to explore anticancer mechanism of drugs. The optimal extraction process of total phenolic acids in *Phyllanthus emblica* was extraction at 50°C for 2 h with 50% ethanol. The total phenolic acids with different concentrations have remarkable inhibiting effects on human Hepatocarcinoma cell SMCC-7721. The growth inhibitory rate of cells in test group is on the rise and depends on density with the increasing of medicine concentrations. Flow cytometry testing results show that the total phenolic acids in Fructus Phyllanthi can stop human Hepatocarcinoma cell SMCC-7721 in S phase and the effect is obvious. Conclusion the total phenolic acids in Fructus Phyllanthi have significant inhibiting effects for the proliferation of Hepatocarcinoma cell. It can induce the cell apoptosis, and has better anticancer effects.

Keywords: Phenolic acids, Hepatocarcinoma Cell, MTT, Flow Cytometry.

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Introduction

Fructus Phyllanthi is the fruit of Euphorbiaceae, *Phyllanthus* and *Phyllanthus emblica* L. [1], with aliases of *Phyllanthus Emblica*, Emblic Leafflower (Tibetan medicine). It is the deciduous shrub or a small tree and that mainly originates from Yunnan, Sichuan, Guangdong and Guangxi, being harvested after fruit ripening in Autumn, and then dried after boiling water scalding.

Fructus Phyllanthi contains polyphenols (including tannins etc) [2], Organic acid [3], flavonoid [4], reducing sugar, polysaccharide [5], terpene [4], sterols, volatile oil, Vitamin, SOD, Protein, Amino acid, Lactone, Coumarin, Alkaloid, thiamine and microelement such as Zn, Mn, Fe, Rb, Sr and Se etc [6]. Among them, the main contents include more contents of tannins, flavonoid, polysaccharide and Vitamin C.

Up to now, modern pharmacological researchers at home and abroad have found that Fructus Phyllanthi is equipped

with antibacterial and anti-inflammatory properties, and the evident inhibiting effects on various anticancer cells [7, 8]. Meanwhile, some researchers found that total phenolic acids are also equipped with anticancer property [9]. Thus this experiment obtains the total phenolic acids by separating them from the Fructus Phyllanthi, and then applies them in the human Hepatocarcinoma cell SMCC-7721, and studies the killing effects on total phenolic acids in Fructus Phyllanthi on anticancer cells. MTT method refers to a method of detecting cells survival and growth. The principle of MTT is that mitochondrial succinate dehydrogenase in living cells can make the exogenous determination of MTT reduction for water insoluble violet crystal Formazan and deposition in cells. And the dead cells do not have this feature.

Material and Methodology

Materials

Agilent 2100, DAD G1315A (Agilent Technology Co., LTD). Human Hepatocarcinoma cell SMCC-7721 (Liaon-

ing University of Traditional Chinese Medicine), Fructus Phyllanthi (batch number: 20100502, Anhui Guoxin Chinese Herbal Medicine Co., LTD, identified as the genuine medicine by professor Zhai Yanjun in Liaoning University of Traditional Chinese Medicine), MTT (produced by Maerican GIBCO company), CTX(batch number: 09040721, produced in Jiangsu Hengrui Pharmaceutical Company), FLOW CYTOMETRY (BD FACSAria II).

Methodology

Quantification methods for total phenolic acids

The content of total phenolic acids was determined to use gallic acid as a standard. 2mL of stock solution of each extract was accurately drawn and placed in a 50mL volumetric flask, the flask was then added sequentially with 5mL 50% ethanol solution, 1mL 0.3% sodium dodecyl sulfate and 1mL mixed chromogenic reagent, i.e. 0.6% potassium ferricyanide and 0.9% ferric trichloride (10:9), shaken well, and placed in a dark place for 15 min, next, the flask was diluted to the mark with 0.1 mol/L hydrochloric acid, followed by the measurement of absorbance of the solution. Standard curve formula was employed to calculate the content of total phenolic acids in each extract.

Single factor test

5 aliquots of crushed *Phyllanthus emblica* powder were weighed, each aliquot had a weight of 5g, after adding 100mL of 70% ethanol solution, the powders were extracted under reflux in water baths at 30 °C, 40 °C, 50 °C,

60 °C and 70 °C for 1 h respectively, and the optimal extraction temperature was screened taking total phenolic content as an index.

5 aliquots of crushed *Phyllanthus emblica* powder were weighed, each aliquot had a weight of 5g, after adding 100mL of 70% ethanol solution, the powders were extracted under reflux in a 50 °C water bath for 1h, 2h, 3h, 4h and 5h respectively, and the optimal extraction time was screened taking total phenolic content as an index.

5 aliquots of crushed *Phyllanthus emblica* powder were weighed, each aliquot had a weight of 5g, after adding 100mL of 10%, 30%, 50%, 70% and 90% ethanol solutions, the powders were extracted in a 50 °C water bath for 2h respectively, and the optimal extraction concentration was screened taking total phenolic content as an index.

5 aliquots of crushed *Phyllanthus emblica* powder were weighed, each aliquot had a weight of 5g, after adding 50% ethanol solutions in ratios of 1:10, 1:20, 1:30, 1:40 and 1:50, i.e. with volumes of 50mL, 100mL, 150mL, 200mL and 250mL, the powders were extracted under reflux in a 50 °C water bath for 2h, respectively, and the optimal solid-liquid ratio was screened taking total phenolic content as an index.

Orthogonal test

Orthogonal experiment was carried out according to Table 1.

Table 1. The methods of orthogonal experiment

Level	Temperature (A)/°C	Time (B)/h	Ethanol concentration (C)/%	Solid-liquid ratio (D)/g/mL
1	40	1	30	1:20
2	50	2	50	1:30
3	60	3	70	1:40

HPLC determination of the content of gallic acid in extract

5 aliquots of 5 g of crushed *Phyllanthus emblica* powder were weighed, added with 100mL of 70% ethanol solution, and extracted under reflux in a 50 °C water bath for 3h respectively, total phenolic acids content was calculated, and quantified by HPLC with gallic acid as a standard.

The conditions of chromatographic: Hypersil C₁₈ (250mm×4.6mm, 5µm); H₂O:CH₃OH:phosphate 95:5:-0.05;1.0mL/min; 270nm; 30 °C.

Cell Culture

Human Hepatocarcinoma cell SMCC-7721 is cultivated in culture media which contains 10% concentration of calf

serum, under 37 °C, saturated humidity, and 5% concentration of carbon dioxide. When cells cover 80% of the cell wall, passage begins, and then extracts cells in log phase of the third generation and planks them to conduct the test.

Experimental group

Put the human Hepatocarcinoma cell SMCC-7721 in log phase into 96 wells plates as per 5000 cells for each well, with 100µL of each well. And then add the configured Fructus Phyllanthi total phenolic acids into the previous finished 96 wells plates. The final concentrations of drugs in each well is 8.39µg/mL, 16.78µg/mL, 33.5µg/mL, 67.1µg/mL and 83.9µg/mL respectively, however, there is no drug added into the blank group. Move the culture plate into CO₂ incubator, and cultivate for 24h, 48h and 72h respectively under 37 °C and 5% concentration of car-

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bon dioxide, make MTT stain after each time period, and measure absorbance under 492nm wave length, and then calculate inhibiting rate according to the following formula.

Cell growth inhibiting rate (IR%) = $(OD_{\text{blank group}} - OD_{\text{sampling group}}) / OD_{\text{blank group}} \times 100\%$

Flow cytometry test the effects of *Fructus Phyllanthi* total phenolic acids on the cell cycles and apoptosis of human Hepatocarcinoma cell SMCC-7721

The experiment is divided into three groups which are low-dose group, and high-dose group and blank control group. Among them, the drug concentration is 167.8 $\mu\text{g}/\text{mL}$ in low-dose group and 419.5 $\mu\text{g}/\text{mL}$ in high-dose concentration, and there is no drug in blank group. Collect cells after 36h, and then make these collected cells into single cell suspension, next, add the ethanol with 70% concentrations, and fixing them for 12h. After that re-suspended cells by PBS, and stain by propidium iodide.

When it is finished, put the sample into sample room of flow cytometry, and check the red fluorescence when the,

excitation wavelength is reach 488nm by using flow cytometry and check the situation of light scattering at the same time. Analyze the results with cell cycle fitting software ModFit. Remove the conjoined cells and display through FL2-w and FL2-A during analysis.

Statistical Treatment

Calculate the half effective concentration (IC₅₀) by Logit special software, present the date with ($\bar{x} \pm S$) and Conduct the single factor variance analysis for the date in each group by using SPSS11.5 software.

Results

The Standard Curve

Take the gallic acid standard article as the abscissa and the absorbance as the ordinate; we can get the linear relationship between absorbance and concentrations. See Fig. 1

The optimal extraction process of total phenolic acids in *Phyllanthus emblica* was extracted at 50°C for 2 h with 70% ethanol. See Fig. 2

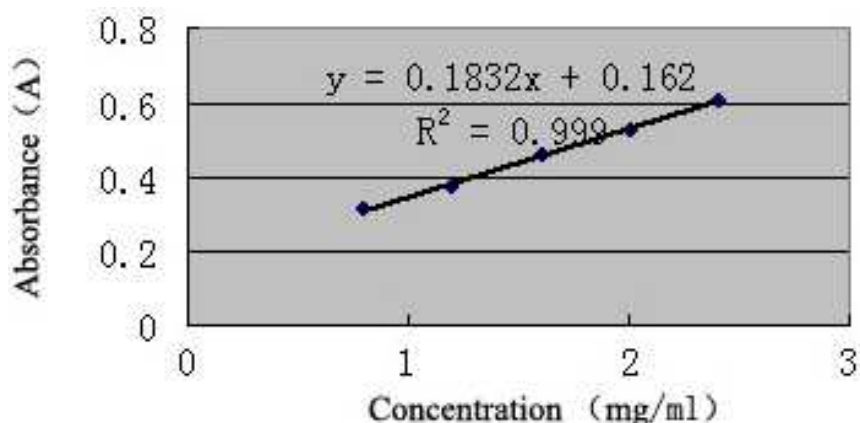
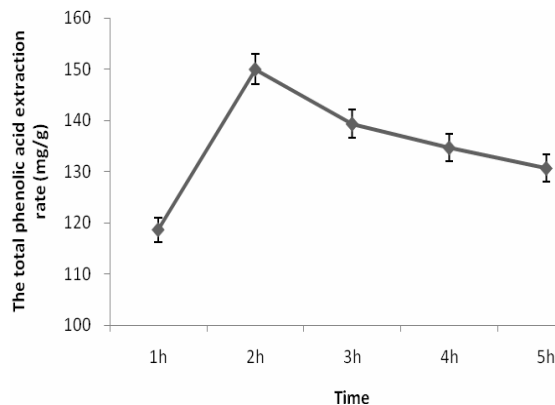
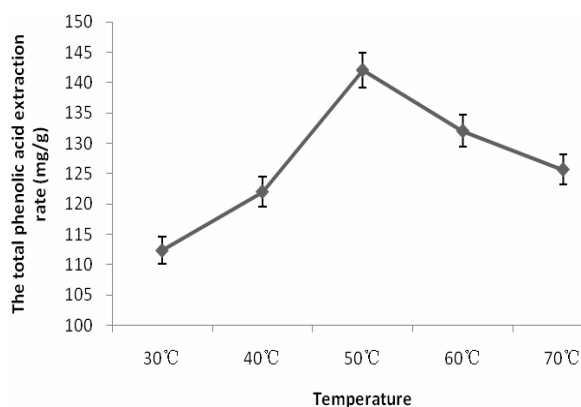


Figure 1. Gallic acid standard curve

From the curve in the figure, we can get the regression equation of Gallic acid content ($\mu\text{g}/\text{mL}$) and absorbance: $Y=0.1832X+0.162$ $R=0.9991$, which indicating that the linear relationship between the two is good.

Single factor test results



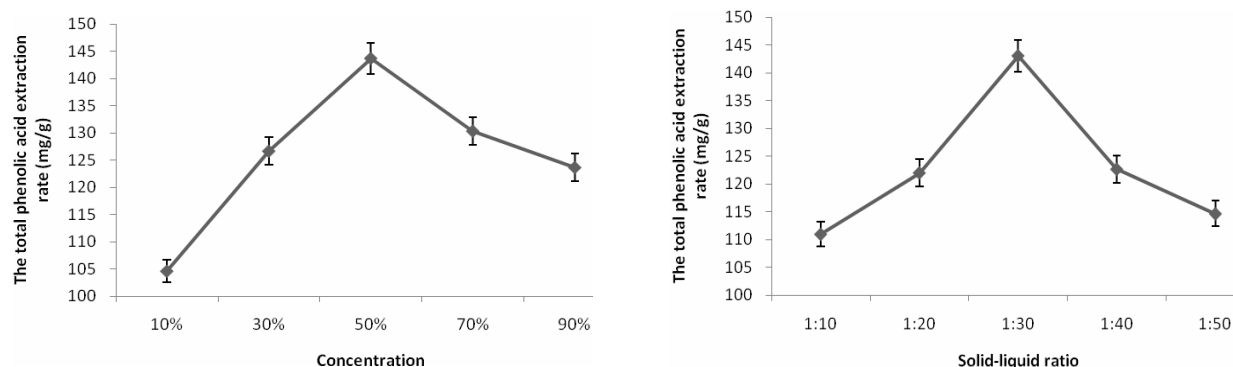


Figure 2. The results of best extraction condition

Orthogonal experimental results

Table 2. The result of orthogonal experiment

	Temperature	Time (h)	Ethanol concentration (%)	Solid-liquid ratio (1:n)	Experimental results (mg)
1	40	1	30	20	117.32
2	40	2	50	30	132.11
3	40	3	70	40	126.14
4	50	1	50	40	129.16
5	50	2	70	20	146.32
6	50	3	30	30	139.22
7	60	1	70	30	128.11
8	60	2	30	40	134.12
9	60	3	50	20	132.29
k1	125.19	124.863	130.22	131.977	
k2	138.233	137.517	131.187	131.147	
k3	131.507	132.55	133.523	129.807	
R	13.043	12.654	3.303	3.34	

Orthogonal experiment results are shown in Table2.

ANOVA results

Table 3. The results of variance analysis

Factor	Sum of squared deviations	Degrees of freedom	F ratio	F critical value	Significance
Temperature	255.277	2	14.751	19	<1
Time	243.859	2	14.091	19	<1
Ethanol concentration	17.306	2	1.000	19	<1
Solid-liquid ratio	17.233	2	0.996	19	<1
Error	17.23	2			

It could be seen by visual analysis and ANOVA that the optimal extraction method was A2B2C3D1, i.e. solid-liquid ratio of 1:30, extraction under reflux at 50 °C for 2h with 50% ethanol. The results are shown in Table3.

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HPLC quantitation results

The results are as shown in the Fig.3.

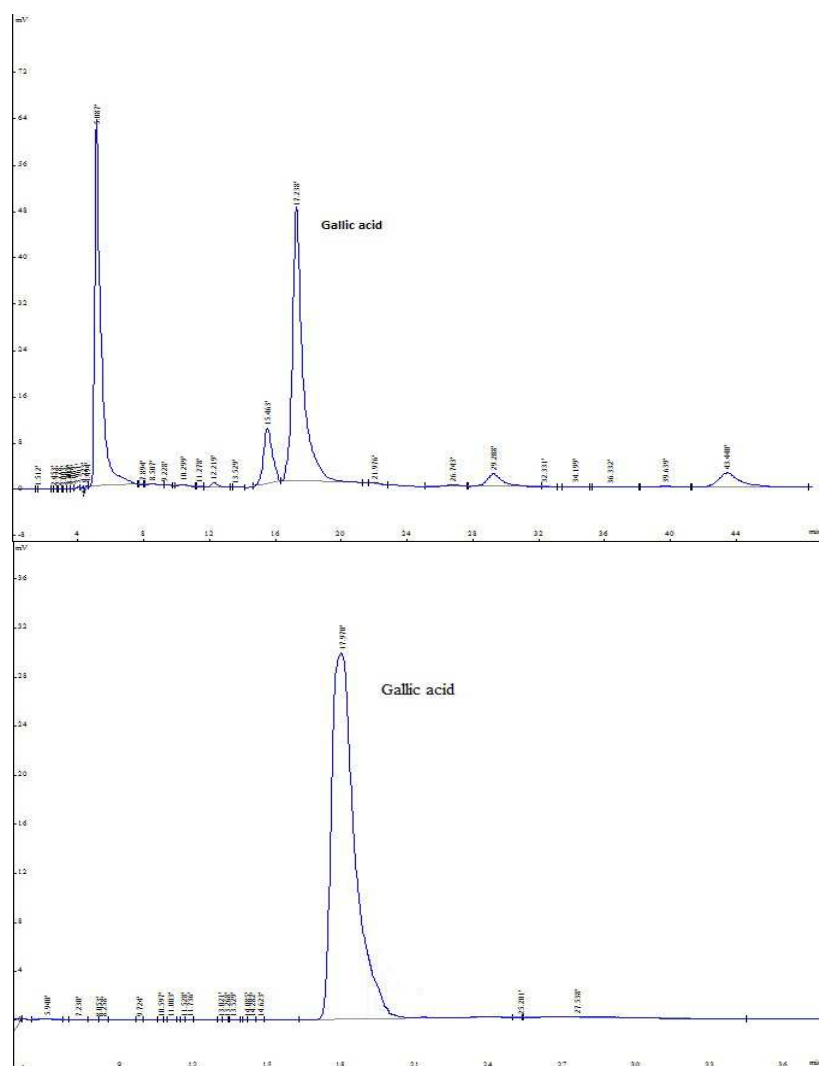


Figure 3. HPLC of Gallic acid and total phenolic acids in *Phyllanthus emblica*

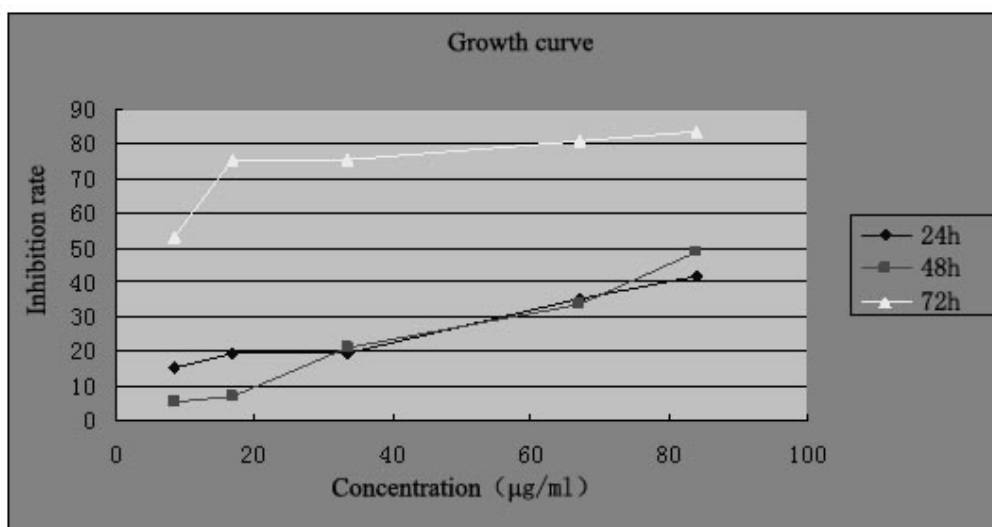


Figure 4. Growth Curve Graph of Fructus *Phyllanthi* total phenolic acids for human Hepatocarcinoma cell SMCC-7721
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The yield of total phenolic acids in *Phyllanthus emblica* was 151.23 mg/g when prepared according to the optimal extraction method, of which Gallic acid content was 10mg/g.

The growth curve chart at 24h, 48h and 72h, when acting Fructus Phyllanthi total phenolic acids on the human Hepatocarcinoma cell SMCC-7721

From Fig.4, it can be seen that during the time period from 24h to 48h, there is no rise of inhibiting rate, but the

inhibiting rate almost reaches to 80% at 72 h, which possibly because the nutrient in culture medium has exhausted after long-time cultivation, and the cells quality decrease. We can observe from the figure that the inhibiting rate presents upward trend along with the increase of drug concentration, which indicates that inhibition effects of *Fructus phyllanthi* total phenolic acids on human Hepatocarcinoma cell SMCC-7721 is concentration dependent.

The effects of Fructus Phyllanthi total phenolic acids on cell cycle and apoptosis of human Hepatocarcinoma cell SMCC-7721

Table 4. DNA content distribution in cell cycle

drug concentration calculate as index component rutin (µg/mL)	cell cycle distribution (%)		
	G0/G1	S	G2/M
0 (blank comparison)	75.05	17.27	7.68
272 (low)	63.65	20.73	15.72
584 (high)	62.62	37.38	0

Combine Table4 with Fig.4, it can be seen that when comparing with the blank group, DNA content in G0/G1 phase decrease for low-dose group .but the contents in S phase largely increase, and have risen by 10.49% when comparing with blank group in S phase. In M phase however, the content is 0, which indicates that *Fructus Phyllanthi* total phenolic acids cause SMMC-7721 cells stop in S phase, and make them apoptosis accordingly.

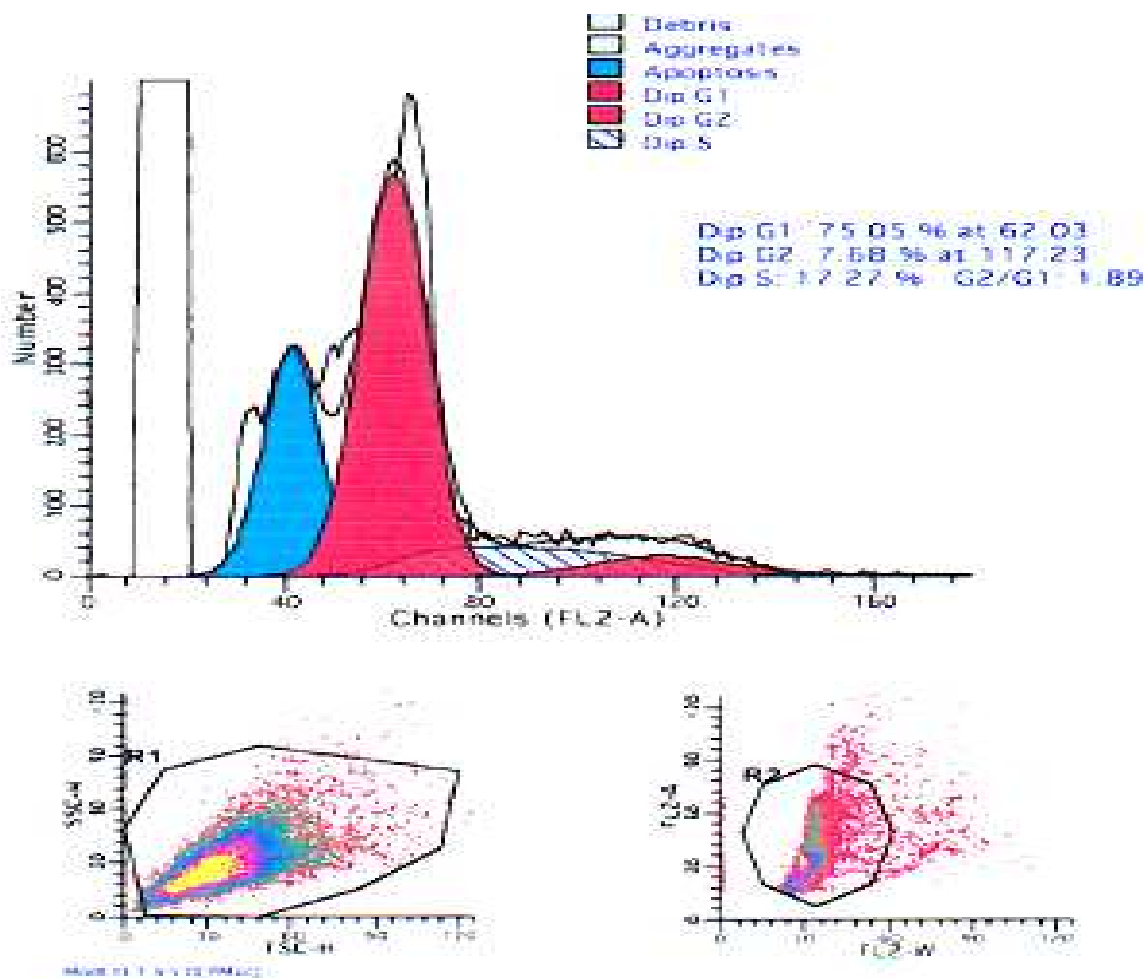


Figure 3. Blank group

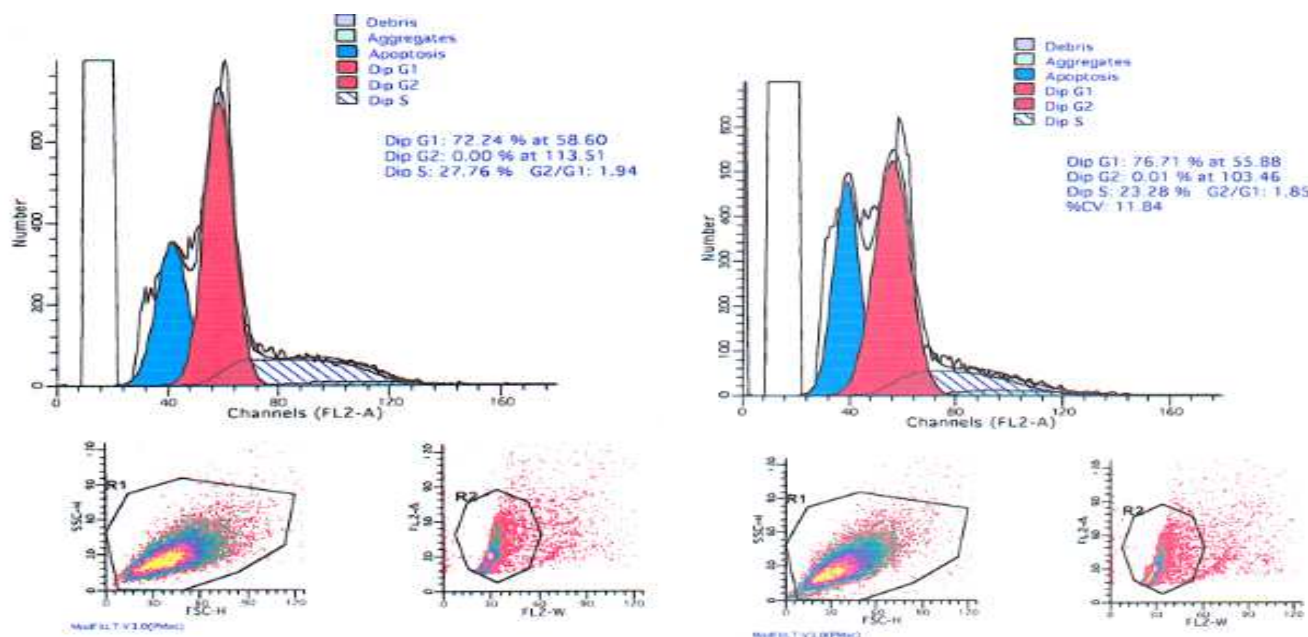


Figure 5. Flow cytometry analysis after *Fructus Phyllanthi* total phenolic acids in low-dose (left) and high-dose (right) group acting on cells

Compared with blank group, DNA contents in G₀/G₁ phase slightly rise, but all largely rise in S phase, and have risen by 6.1% when comparing with blank group in S phase, and the content in M phase is 0, which indicates that *Fructus Phyllanthi* total phenolic acids cause SMMC-7721 cells stop in S phase, and make them apoptosis accordingly, however, the effect in high-dose group is worse than in low-dose group. See Fig. 5.

DNA contents in S phase have remarkable increase in this experiment. All *Fructus Phyllanthi* total phenolic acids extracts with different concentrations can lead SMMC-7721 cells to block in S phase, and thus hinder cells enter into G₂/M phase, and also affect the cells enter into G₀/G₁ phase, which make cell cycle process blocked. See Fig. 5.

Discussion

Preliminary experiments prove that *Fructus Phyllanthi* contains large quantity of phenolic acid compound. And some researchers at home and abroad have founded that phenolic acid compound have antibacterial and anti-inflammatory properties [10-17]. So this experiment aims at obtaining total phenolic acids extracted from *Fructus Phyllanthi* and determines their inhibiting rate for human Hepatocarcinoma cell SMCC-7721 and their effects on cell cycle by MTT and flow cytometry method.

Phyllanthus emblica mainly contains phenolic acids, whose polarity is relatively high, so it is reasonable to use 50% ethanol in the extraction. Optimal extraction process of total phenolic acids in *Phyllanthus emblica* is determined finally, which is conducive for the future devel-

opment and application of the product, and plays a guiding role of large scale production.

Through MTT method, we can see that *Fructus Phyllanthi* total phenolic acids can inhibit the growth of human Hepatocarcinoma cell SMCC-7721, and present concentration dependent, which inhibiting rate increase accordingly with the increase of concentration. From the growth curve figure of human Hepatocarcinoma cell SMCC-7721, we know that IC₅₀ of *Fructus Phyllanthi* total phenolic acids for human Hepatocarcinoma cell SMCC-7721 is around 167.8µg/mL, so in the experiment that determine their inhibiting rate for human Hepatocarcinoma cell SMCC-7721 and their effects on cell cycle, the final drug concentration in low-dose group is 167.8µg/mL.

The cell generation cycle can be classified into five phases: stationary phase (G₀), DNA presynthetic phase (G₁), DNA synthesis stage (S), post-synthetic phase (G₂), mitosis phase (M). There are two critical check points in the common cell cycle: one is that whether the cell generation cycle can process smoothly that mainly depends on whether this two key checkpoints G₁/S transition and G₂/M transition can be done well. The cells analyze kinds of signals and DNA damage condition in the checkpoint of G₁/S transition, after that, cells will determine whether need to enter into the next phase; the other is G₂/M transition checkpoint, common wisdom has it that DNA repair accomplished at this phase, and then determine whether the cell shall enter in the mitotic phase. The important reason which cause cell canceration is possibly that cell cycles lose control thus the cell generations are out of control [18].

This experiment proves that Fructus Phyllanthi total phenolic acids can block SMMC-7721 cells in S phase, and this block effect is significant, which almost no cells enter into the next G₂/M phase. Because the flow cytometry can't distinguish the G₂ phase from the M phase both of which have same DNA contents, to determine that whether cells in G₂ phase have entered into the mitotic phase is need further research.

To sum up, Fructus Phyllanthi total phenolic acids have obvious inhibiting effects on the generation of human Hepatocarcinoma cell SMCC-7721, which can cause the cell apoptosis. Compared with the other chemotherapy drug, it has the better anticancer effect, with smaller side effects. Further study its effective molecular mechanism have remarkable significance for the future clinical application and products development.

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