INTRODUCTION
Liver cancer is a common malignant tumor. Early surgical resection is the key to its treatment, but most patients are already at the advanced stage when diagnosed. The overall curative effect is poor. Therefore, finding new anti-hepatoma drugs has become a research focus in the treatment of liver cancer. Semen litchi is a dry mature seed of litchi, which belongs to Sapindaceae. It is sweet, slightly bitter, and has the effects of promoting the circulation of blood dissolving lumps, resisting coldness and relieving pain. Modern research showed that semen litchi had many pharmacological effects, such as blood sugar reduction, blood lipid regulation, liver protection and anti-oxidation. The rapid growth of cancer tissue has to be accompanied by the growth of new blood vessels so as to ensure sufficient nutrient supply and maintain the exuberant metabolism of liver cancer cells and their metastatic spread. Therefore, it is an important anticancer strategy to inhibit the formation of blood vessels in liver cancer and cut off the life-blood of liver cancer growth and metastasis. Experiments have shown that vascular endothelial growth factor (VEGF) is expressed strongly in hepatoma cells. VEGF is the most well-known cytokine that promotes vascular endothelial growth. The expression of matrix metalloproteinase-9 (MMP-9) in hepatocellular carcinoma is significantly higher than that in adjacent tissues, and the imbalance of MMP-9 expression is closely related to the invasion and metastasis of hepatocellular carcinoma. At present, there are few studies on the effect of semen litchi drug serum on the expression of VEGF and MMP-9 in human hepatoma HepG2 cells. The objective of this study was to investigate inhibitory effect of semen litchi drug serum on proliferation of human hepatoma HepG2 cells and its effect on the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9).

METHODOLOGY
This study was conducted at the College of Pharmacy, Guangxi University of Chinese Medicine, China, from
June 2017 to January 2018. The research was conducted after approval from the Committee on Animal Research and Ethics. Eight healthy male New Zealand rabbits weighing approximately 1.5 kg were randomly divided into the control group (administered with an equal volume of normal saline), the semen litchi high dose group (12.0 g/kg), the semen litchi medium dose group (6.0 g/kg) and the semen litchi low dose group (3.0 g/kg). Administration by gavage was conducted twice a day with an interval of 12 hours. Fasting without food (water allowed) was conducted 4 hours before administration for 3 consecutive days. One hour after the last administration by gavage, 3% sodium pentobarbital was used for anesthesia, and blood of the carotid artery was collected, left for 4 hours at 4°C, centrifuged at 3000 r/min for 15 minutes, and the serum was aseptically separated. After being inactivated at 56°C for 30 minutes, filtered and sterilised by 0.20 μm microporous filtering membrane, semen litchi drug serum was prepared into the control group (0 g/kg), high-dose group (12.0 g/kg), medium-dose group (6.0 g/kg) and low-dose group (3.0 g/kg), and stored at -20°C until use.

After HepG2 cells were resuscitated, they were cultured in DMEM nutrient solution (containing 10% calf serum, penicillin 100 U/mL, and streptomycin 100 μg/mL) in a 37°C 5% CO₂ incubator. The cells were adherent to the wall, each generation created in every 2-3 days, and cells in exponential growth phase were used for experiments.

Inhibitory effect of semen litchi drug serum on HepG2 cell proliferation was detected by MTT Assay. HepG2 cells in logarithmic growth phase were inoculated in a 96-well plate at a cell concentration of 3 x 10⁴ /mL, 100 μl of each well. The cells were adherently grown for 24 hours before a final concentration of 30% of the drug-containing serum medium acted on the cells. Four duplicate wells were set for the four groups of concentrations of the control group, the high dose group, the medium dose group and low dose group. The final concentration of 5 g/LMTT solution was added after 72 hours of culture. After incubation for 4 hours in the incubator, the culture medium was removed. DMSO was added to dissolve the crystals. The absorbance at 570 nm was measured with a microplate reader to calculate the inhibitory effect of semen litchi drug serum on HepG2 cells after 24, 48 and 72 hours. Calculation formula: cell inhibition rate (%) = (absorbance optical density (OD) value of the control group - absorbance OD value of the treated group) / absorbance OD value of the control group x 100%.

The expression of VEGF and MMP-9 mRNA in HepG2 Cells were detected by real-time PCR. According to the cell count results, the cell density was adjusted to 1 x 10⁶ cells/mL, and concentrations of the control group, the high-dose group, the medium-dose group and the low-dose group acted on HepG2 cells for 48 hours, respectively. Total mRNA kit from Takara Company was used to extract total cellular RNA. cDNA was prepared as described in instruction of the Prime Script®RT Kit with gDNA Eraser (Perfect Real Time) reverse transcription kit. Real-time PCR amplification was conducted using cDNA as template and GADPH as internal reference. The VEGF upstream primer: 5'-TGGAGTGTGTGCCCATGAG-3'; the downstream primer: 5'-TGCATTCAACATTGTGCTGTAG-3'; and the amplified fragment length was 117 bp. MMP-9 upstream primer: 5'-TCCCTG-GAGACCTGAGAACC-3'; downstream primer: 5'-CG-GCAAGCTTTCCCGAGTAGTTT-3'; and the amplified fragment length was 307 bp. GAPDH upstream primer: 5'-GCACCGTCAAGGCTGAGAC-3'; downstream primer: 5'-TGGTGAGACCGCGAGTGA-3', and the amplified fragment length was 138 bp. The reaction system was 2×super real pre mix plus 10 μL, 0.8 μL of each of the upstream and downstream primers (concentration: 10 μmol/L), 2 μL of cDNA (stock solution), 1 μL of ROX reference dye (50×), and 6.4 μL of RNase free dH₂O. The total system was 20 μL. The reaction conditions were as follows: pre-denaturation at 95°C, 30s for one reaction cycle, 95°C for 10s, 60°C for 34s, a total of 40 cycles; 55°C -95°C for 30s, a total of 81 reaction cycles. After the end of PCR amplification, the analyser displayed the standard curve, amplification curve and melting curve. The amplification factor was calculated using the2-ΔΔct method.

The expression of VEGF and MMP-9 protein in HepG2 cells were detected by western blot. After treatment of HepG2 cells for 48 hours with concentrations of the control group, high-dose group, medium-dose group and low-dose group, strong RIPA (premixed with PMSG 100:1) lysate was used to extract total protein of cells from each group. BCA kit was used to determine protein concentration. Twenty μg total protein was used to prepare protein sample for loading, 10% SDS-PAGE gel electrophoresis, PVDF membrane transfer, membrane closure for 2 hours, and primary antibody incubated overnight at 4°C. The membrane was washed 3 times with TBST, and secondary antibody (HRP-labelled goat anti-mouse antibody) was added and incubated at room temperature for one hour. The membrane was washed three times (10 minutes/time) and exposed to light by chemiluminescence. β-actin was used as internal reference.

SPSS 22.0 software was used for statistical analysis of the data. Measurement data was expressed with mean ±SD. One-way ANOVA analysis of measurement data was used for comparison among groups. The SNK test was used for the mutual comparison between multiple groups. The p < 0.05 was considered to be statistically significant.
RESULTS
The semen litchi low-dose group, medium-dose group, and high-dose group acted on the HepG2 cells for 24, 48, and 72 hours, respectively. The results showed that the inhibitory effect of semen litchi drug serum on the proliferation of HepG2 cells significantly increased with the increase of drug concentration, which was dose-time dependent. The inhibitory rate of HepG2 cell proliferation in the semen litchi low-dose group, medium-dose group, and high-dose group was significantly different from that in the control group (all p <0.001, Table I).

After acting on HepG2 cells for 48 hours, the expression levels of VEGF and MMP-9 mRNA in HepG2 cells after 48 hours of treatment by semen litchi low-dose group, medium-dose group, and high-dose group were significantly lower than those in the control group (all p <0.001, Table II); and with the increase of semen litchi drug serum concentration, the inhibitory effect on the expression of VEGF and MMP-9 mRNA became more obvious.

After acting on HepG2 cells for 48 hours, the relative expressions of VEGF and MMP-9 protein in semen litchi low-dose group, medium-dose group, and high-dose group were lower than those in control group (all p<0.001); and with the increase of concentration, semen litchi drug serum's inhibition of VEGF, MMP-9 protein expression increased, as shown in Figure 1.

DISCUSSION
The use of medicine-containing serum instead of crude extracts as a medicine-carrier can effectively avoid the disadvantages brought by direct use of crude extracts of traditional medicines, such as excessive ingredients, difficulty in determining active ingredients, inconsistent in vitro and in vivo test results and efficacies and many other problems.9,10 Therefore, in vitro experiments using drug-containing serum can simulate the actual process of pharmacological effects produced by the agent in vivo, which is theoretically more scientific and authentic.

The occurrence of tumors is associated with abnormal cell proliferation and differentiation and the inhibition of its apoptosis.11,12 Inhibiting tumor proliferation and promoting tumor cell apoptosis are important methods for tumor prevention and treatment. Traditional Chinese medicine semen litchi has the effects of promoting the circulation of qi, dissolving lumps, resisting coldness and relieving pain. Studies have confirmed that semen litchi has many pharmacological effects.13 In addition, semen litchi has a high content of crude fiber, minerals, essential amino acids, and essential fatty acids, as well as litchi acid with a good protective effect on the cardiovascular system. It is a good source of nutrition for the development of elderly health foods. Studies have shown that litchi seed extracts had anti-prostate cancer effect.14 Other research has shown that litchi seed extract could suppress epidermal growth factor receptor signalling and inhibited non-small cell lung cancer cell growth.15

In this experiment, serum pharmacological methods were used to observe the effect of semen litchi drug serum on the proliferation inhibition rate of human hepatoma HepG2 cells. The results of this study indicate that semen litchi drug serum has a good effect on inhibiting proliferation of HepG2 cells, and showing a dose-time dependent effect. This conclusion was similar to the result of Wang et al., which indicated that litchi fruit pericarp extract demonstrated a dose- and time-dependent inhibitory effect on cancer cell growth.16

Table II: The effect of semen litchi drug serum on the expression of VEGF mRNA and MMP-9 mRNA in HepG2 cells after 48h of treatment (n=4).

<table>
<thead>
<tr>
<th>Groups</th>
<th>VEGF mRNA</th>
<th>MMP-9 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>p-value</td>
</tr>
<tr>
<td>Control group</td>
<td>0.972 ±0.163</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Semen litchi low-dose group</td>
<td>0.810 ±0.335</td>
<td>0.700 ±0.346</td>
</tr>
<tr>
<td>Semen litchi medium-dose group</td>
<td>0.252 ±0.171</td>
<td>0.401 ±0.230</td>
</tr>
<tr>
<td>Semen litchi high-dose group</td>
<td>0.080 ±0.074</td>
<td>0.127 ±0.084</td>
</tr>
</tbody>
</table>

Table I: The effect of semen litchi drug serum on the proliferation inhibition rate of HepG2 Cells (n=4).

<table>
<thead>
<tr>
<th>Groups</th>
<th>OD value</th>
<th>Inhibitory rate (%)</th>
<th>OD value</th>
<th>Inhibitory rate (%)</th>
<th>OD value</th>
<th>Inhibitory rate (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.54±0.01</td>
<td>0.00 ±0.00</td>
<td>0.53±0.01</td>
<td>0.00 ±0.00</td>
<td>0.54±0.02</td>
<td>0.00 ±0.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Semen litchi low-dose group</td>
<td>0.50±0.05</td>
<td>7.41 ±0.07</td>
<td>0.49±0.01</td>
<td>7.55 ±0.23</td>
<td>0.49±0.01</td>
<td>9.26 ±0.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Semen litchi medium-dose group</td>
<td>0.47±0.02</td>
<td>12.90 ±0.28</td>
<td>0.44±0.02</td>
<td>16.98 ±0.62</td>
<td>0.44±0.01</td>
<td>18.52 ±0.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Semen litchi high-dose group</td>
<td>0.40±0.03</td>
<td>25.93 ±0.69</td>
<td>0.38±0.02</td>
<td>28.30 ±1.15</td>
<td>0.36±0.02</td>
<td>33.33 ±1.39</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 1: The effect of semen litchi drug serum on the expression of VEGF and MMP-9 in HepG2 cells after 48h of treatment.
(a) Control group; (b) Semen litchi low-dose group; (c) Semen litchi medium-dose group; (d) Semen litchi high-dose group.
VEGF is the most potent angiogenic factor in vivo. It is highly expressed in liver cancer, and its receptors (VEGFRs) play an important role in the growth, metastasis and recurrence of liver cancer. The major reason for the difficulty of treating primary liver cancer is invasion and metastasis. Matrix metalloproteinases (MMPs) are a family of endopeptidases that degrade ECM. They play an important role in cell movement, basement membrane degradation and lumen formation during angiogenesis and are closely related to the invasion and metastasis of primary liver cancer. In addition, studies have shown that vascular endothelial growth factor stimulates endothelial cells to produce MMPs, or activates the matrix-degrading cascade reaction by modulating the balance of MMPs and their inhibitors tissue inhibitors of metalloproteinases (TIMPs), thus allowing tumor cells to penetrate more easily and affecting the infiltration and metastasis of tumors. In the MMP family, MMP-9 is a common tumor marker that reflects the invasion, metastasis, and recurrence of primary liver cancer. At present, the specific molecular mechanism of anti-hepatocarcinoma effect of semen litchi drug serum is not yet known.

The results showed that the expression levels of VEGF and MMP-9 mRNA and protein were lower in HepG2 cells treated with semen litchi drug serum in the low, medium and high dose groups than those in the control group, and with the increase of concentration, semen litchi drug serum's inhibition of the expression of VEGF and MMP-9 mRNA and protein increased. It suggested that the semen litchi drug serum had a certain inhibitory effect on the expression of VEGF and MMP-9 mRNA and protein. This confirmed the inhibitory effect of semen litchi drug serum on the expression of VEGF and MMP-9 in HepG2 cells from the level of gene and protein expression.

One study had shown that litchi seed extracts caused dose-dependent apoptosis of HepG2 cells through cell cycle arrest at G0/G1 phase, thus preventing cells entering S or G2/M phase. In view of this, the authors speculated that semen litchi drug serum might inhibit cell proliferation by G0/G1 phase arrest in HepG2 cells. It could be further studied in the future to confirm it.

It is worth mentioning that, at present, studies on litchi anti-tumor have shown good anti-tumor activity only in vitro. The research and development of litchi antineoplastic drugs is still in the laboratory evaluation stage. There are no litchi antineoplastic drugs on the market, and they have yet to be further developed.

**CONCLUSION**

In short, semen litchi drug serum can inhibit the proliferation of hepatoma cells in vitro. The anti-hepatoma effect of semen litchi drug serum may be exerted through down-regulating the expression of VEGF and MMP-9 and inhibiting angiogenesis of hepato-cellular carcinoma.

**REFERENCES**


