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Characterization and anti-tumor effect of shark liver extraction

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Shark liver oil (SLO) has been widely used as a food supplement to boost human immunity. The extraction method of shark liver oil is immature. In this study, four different extraction methods of shark liver oil were tried, the effect of the solid-liquid ratio, quality of the enzyme, reaction pH and temperature on SLO extraction rate have been discussed. Infrared spectroscopy, gas chromatography and high performance liquid chromatography was used to detect the main composition of SLO, and U14 tumor bearing mice were used to explore anti-tumor effect of SLO. Our results show that alkaline protease extraction method was the best with an extraction rate of 30.15%, and the solid-liquid ratio, quality of the enzyme, reaction pH and temperature was 1:2, 2% of the liver quality, 9, 3.5 h, 48°C respectively. Characteristic peak of alkoxy glycerol, squalene and vitamin A was presented in the IR spectra of SLO, the proportion of alkoxy glycerol, squalene content and vitamin was 35.3, 40.4 and 7.8% determined by gas chromatography (GC) and high performance liquid chromatography (HPLC). The inhibition rate was 54.56.

Key words: Shark liver oil, extraction, characterization, gas chromatography, high performance liquid chromatography (HPLC).

INTRODUCTION

Shark liver oil contains a variety of active ingredients, including vitamins A, vitamin D, and squalene terpenoids (Wang, 2006; Ma et al., 2010). It can activate the reticuloendothelial system, enhance the anti-tumor ability (Wang, 2003), promote the vitality of immune cells, stimulate the regeneration of the immune cells (Huang et al., 2009; Joon-Son Sim et al., 2007), clear intracellular radicals, regulate physiological balancing effect, and also increase the quantity of white blood cells, platelets and lymphocytes (Laurence et al., 2002; Zi et al., 2000).

There is a number of shark liver oil extraction method for instance, the water extraction method is of a low cost and simple method (Bao et al., 2006), which cannot effectively separate the oil, which tightly connect with tissue and is only suitable for small work shop production. Supercritical fluid extraction needs large equipment and has a large cost (Han et al., 2003). The oil extraction method had a low market value. Finally, the ultrasonic extraction is unable to separate the fat composition from the protein (Hu et al., 2002; Romain et al., 2004). Among

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the extraction techniques, the effect of squeeze extraction is poor, which had greater cost, low utilization rate and a serious waste. The increased demanding increasing and the limited source of the shark make the exploration of a high efficient extraction method of shark liver oil critical.

MATERIALS AND METHODS

Chiloscyllium plagiosum was purchased from the Bohai Sea Creatures Germplasm Breeding Engineering Center. NaCl, KOH, methanol, alkaline protease were purchased from Shanghai sangon (Shanghai, China).

The extraction of shark liver oil

Dissect sharks, set the liver tissue into tissue grinder, and add ice normal saline. Alkali extracting, take 20 g shark liver homogenate, add 2-fold mass of distilled water in a magnetic stirrer, 300 rpm the oscillation heated up to 45°C. The pH was adjusted to 9, the oscillation continues to heat up to 80°C, and maintaining the temperature, until the liver homogenate become deep dark and almost non-sticky, add 0.8 g NaCl, 300 rpm, 55°C mix 15 min, 8000 rpm centrifugation 10 min, the supernatant was repeatedly washed, until the pH constant, weigh and records. Accurate weighing shark liver and liver oil quality. Being dependent on the formula below, we can calculate the liver extraction rate (Bao et al., 2006; Han et al., 2003; Hu et al., 2002).

Shark liver oils extraction rates (%) = mass of extracted shark liver oils (g)/mass of shark liver (g) × 100%.

In this experiment, the main influence factors were solid-liquid ratios, enzyme dosage, pH, extracted time and temperature.

First of all, we considered the affection of solid-liquid ratios to the SLO extraction rate. Shark liver tissue homogenate consisted of five groups with 5 g equally. Adding suitable amount of distilled water to each group respectively pro rate of 1:1, 1:1.5, 1:2, 1:2.5, and1:3, and then conduct the experiment depending on the above method then the affection of pH to the SLO extraction rate was studied, shark liver tissue homogenate was divided into five groups of 5 g. Then according to the solid-liquid ratio1:2, adding suitable amount of distilled water, and adjusted the pH to 7.5, 8, 8.5, 9 and 9.5 respectively, the follow steps were conducted according to the aforementioned.

Thirdly, affection of enzyme dosage was discussed. Shark liver tissue homogenate was composed of five groups of 5 g. Then according to the solid-liquid ratio1:2, adding suitable amount of distilled water, and adjusted the pH to 9, added 0.025, 0.050, 0.075, 0.100, 0.125 g alkaline protease respectively, 300 rpm, 45°C extracted 4 h, 8000 rpm centrifugation 15 min, until the pH of the supernatant became unchanged.

The affection of extracted time was determined as well. Shark liver tissue homogenate consisted of five groups of 5 g. Then according to the solid-liquid ratio1:2, adding suitable amount of distilled water, and adjusted the pH to 9, added 0.100 g alkaline protease, 300 rpm, 45°C extracted 2.5, 3, 3.5, 4, 4.5h respectively, 8000 rpm centrifuged 15 min, until the pH of the supernatant became unchanged.

At last, we examined the affection of temperature to the extraction rate. Shark liver tissue homogenate consisted of five groups of 5 g. Then according to the solid-liquid ratio1:2, adding suitable amount of distilled water, and adjusted the pH to 9, added 0.100 g alkaline protease, 300 rpm, 45°C extracted 3.5 h, respectively at 39, 42, 45, 48 and 51°C extracted 3.5 h, 8000 rpm centrifuged 15 min, until the pH of the supernatant became unchanged.

Characterization of SLO

Infrared spectroscopy (IR), gas chromatography (GC), HPLC method was used to detect the main composition of SLO.

Characterization of SLO by IR

Potassium bromide method of infrared sampling was used, and then samples were detected with infrared spectroscopy (Chen et al., 2014).

Characterization of SLO by GC

SLO and 50 g/L KOH were saponified 30 min with the ratio of 1:100 (g/mL). The reaction liquid extracted by mixed solvent of n-hexane and chloroform (4:1) three times. The extract liquid was mixed, and metered volume to 100 ml with n-hexane. Squalene and alkoxy glycerol were detected by GC.

0.8 mg/mL Standard stock solution of squalene were preparation with n-hexane, it was diluted into 0.25, 0.5, 1, 1.5 and 2 mg/mL standard solution, while the concentration of alkoxy glycerol standard solution was 0.1, 0.2, 0.3, 0.4 and 0.5.

Conditions of GC

Chromatographic columns we used was HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm), and the program was 200°C for 1 min, 20°C/min to heat up to 280°C, keep 10 min. Injector temperature and volume were 300°C and 1 μl. Carrier gas flow rate was 0.7 mL/min with a Split ratio at 10:1 (Qi et al., 2010; Tian et al. 2011; Wang, 2003).

Characterization of SLO by HPLC

2 mg/mL Standard stock solution of squalene and alkoxy glycerol were preparation with methanol, they was diluted into 1, 0.5, 1, 1.5, and 2 mg/mL standard solution. The solution was filtered using 0.45 μm membrane.

0.2 mg/mL vitamin A Standard solution was prepared; it was diluted into 0.04, 0.08, 0.12, 0.16 mg/mL standard solution. The solution was filtered by 0.45 μm membrane.

10 mg SLO was dissolved in 10 ml methanol. The solution was filtered by 0.45 μm membrane. Squalene, alkoxy glycerol and vitamin A were detected by HPLC.

The column was XDB (C-18, 5 mm × 25 cm × 5 um), mobile phase was methanol with a flow rate of 0.8 ml/min; the column temperature was kept at 26°C, the detection wavelength was 210, 270 and 320 nm (Dai et al., 2009).

Anti-tumor effect of SLO *in vivo*

The Cervical Carcinoma U14 tumor-bearing mice was randomly assigned into four groups: the negative control group, the cyclophosphamide(CTX) positive control group, the low liver oil group (300 mg/kg) and the high liver oil group (600 mg/kg), 8 mice for each group. Mice were intragastrically successive administered

Table 1. Effect of solid-liquid ratio on the extraction rate of SLO.

Solid-liquid ratio	1:1	1:1.5	1:2	1:2.5	1:3
Liver (g)	4.978	4.986	5.013	5.121	4.994
Shark Liver Oil (g)	1.185	1.275	1.387	1.353	1.209
The rate of shark liver oil extracting (%)	23.81	25.58	27.67	26.43	24.20

Table 2. Effects of pH on the extraction rate of SLO.

initial pH	7.5	8	8.5	9	9.5
Liver (g)	4.613	4.493	4.590	4.136	4.802
Shark Liver Oil (g)	1.098	1.145	1.270	1.174	1.317
The rate of shark liver oil extracting (%)	23.81	25.48	27.67	28.39	27.43

Table 3. Effects of quality of enzyme on the extraction rate of SLO.

Enzyme dosage (%)	0.5	1.0	1.5	2.0	2.5
Liver (g)	5.010	5.043	5.151	5.088	5.029
Shark Liver Oil (g)	1.264	1.384	1.462	1.473	1.460
The rate of shark liver oil extracting (%)	25.23	27.45	28.39	28.96	29.03

with medicines for 15 days 24h later after inoculated tumor. The negative control group was intragastrically administered with distilled water, the CTX (25mg/kg intraperitoneal injected) was set up as the positive drug. At date 16, mice were sacrificed. The animals and the tumor tissue were weighted. The tumor control rate was calculated as the Formula 1.

Tumor control rate = (negative control group-medicine group)/ negative control group (1)

RESULTS AND DISCUSSION

Effects of different factors on the extraction rate of SLO

The extracting rates were calculated respectively; effects of solid-liquid ratio on the extraction rate of SLO were shown in Table 1. As shown in Table 1, when the solid-liquid ratio changed from 1:1 to 1:3, shark liver oil extraction rate was increasing as the solid-liquid ratio increase. When the solid-liquid ratio was 1:2, the extraction rate reached the culmination with the rate of 27.67%. But when the solid-liquid ratio was greater than 1:2, the extraction rate began to decrease. This may be related to the too much solvent added, which result to reduce the concentration of the alkaline protease. Therefore, the most suitable solid-liquid ratio was identified 1:2.

Effects of pH on the extraction rate of SLO were shown in Table 2. As shown in Table 2, when the pH was 7.5, the extraction rate was low. With the pH increasing, shark liver oil extraction rate gradually increased, because raising the initial pH can increase the alkaline protease activity and promote the hydrolysis of the shark liver protein. When the initial pH was 9, the extraction rate reached highest of 28.39%. When the pH exceeded 9, the extraction rate gradually decreased with the increasing of pH. Through the analysis, the optimum pH value should be 9.

Effects of quality of enzyme on the extraction rate of SLO were shown in Table 3. As shown in Table 3, with the increasing of added enzyme dosage, shark liver oil extraction rate also gradually increased. When the enzyme dosage was 2.0%, the extraction rate reached the highest, alkaline protease was saturated. But when enzyme dosage was too high, the mutual hydrolysis of enzyme also increased, which could weaken the alkaline protease hydrolysis of shark liver protein. Considering from the extract yield and economic benefits, the optimum adding amount of alkaline protease should be 2.0%.

Effects of extracted time on the extraction rate of SLO were shown in Table 4. With the increasing of the hydrolysis time, shark liver oil extraction rate increased; because the increase of the hydrolysis time can make shark liver and alkaline protease contact more. But when the hydrolysis time reached 3.5 h, the rate of extension increased slowly. The free fatty acids in shark liver oil

Table 4. Effects of extracted time on the extraction rate of SLO.

Digest time (h)	2.5	3.0	3.5	4.0	4.5
Liver (g)	5.116	5.041	5.453	5.151	4.996
SHARK LIVER OIL (g)	1.348	1.396	1.568	1.493	1.452
The rate of shark liver oil extracting (%)	26.34	27.69	28.76	28.98	29.06

Table 5. Effects of temperature on the extraction rate of SLO.

Temperature (°C)	39	42	45	48	51
Liver (g)	5.015	5.208	4.989	5.008	5.105
Shark Liver Oil (g)	1.297	1.415	1.440	1.510	1.491
The rate of shark liver oil extracting (%)	25.87	27.17	28.86	30.15	29.20

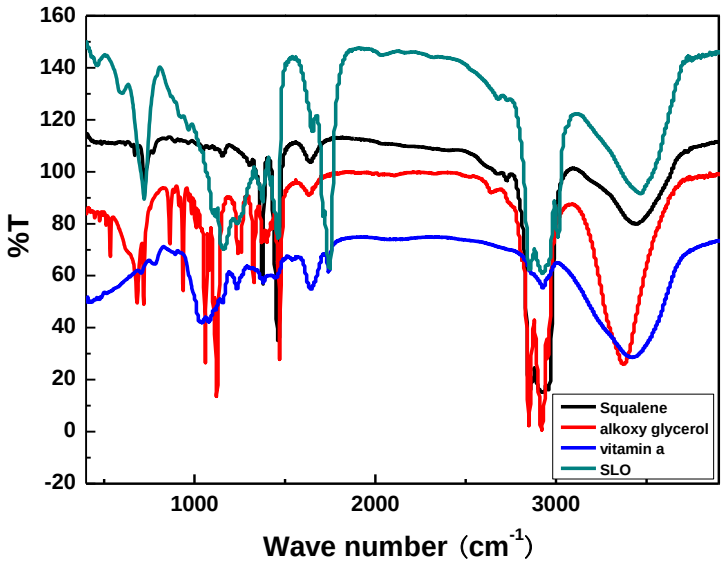

Figure 1. Infrared spectrum.

were oxidized by oxygen in the air when it prolonged exposure to air, which made the shark liver oil color from pale yellow to reddish brown, caused some damage, and affected their quality. Therefore, this experiment extracted time should be 3.5 h.

Effects of temperature on the extraction rate of SLO were shown in Table 5. With the increasing of reaction temperature, the extraction rate of shark liver oil increased, and the enzymatic reaction rate increased within limits. When the reaction temperature reached 48°C, the maximum extraction rate of shark liver oil was 30.15%. Each enzyme has a specific optimum reaction temperature under certain conditions; at the optimum temperature of the enzyme hydrolysis will show the greatest activity. Either too high or too low hydrolysis temperature will affect the kinetic parameters of the enzyme reaction. Therefore, the optimum reaction temperature is 48°C.

SLO characterization

From Figure 1, infrared spectra of SLO contains the characteristic peaks of squalene, alkoxy glycerol and vitamin A, thus it can be preliminarily concluded that there were squalene, alkoxy glycerol and vitamin A in SLO.

The chromatographic analysis results were shown in Figures 1 and 2, the standard curve of spinacene, alkyl oxygen and vitamin A were Y = 7.72203 + 11.37991 * X, Y = -115.21784 + 1851.76871 * X and Y = -157.094 +

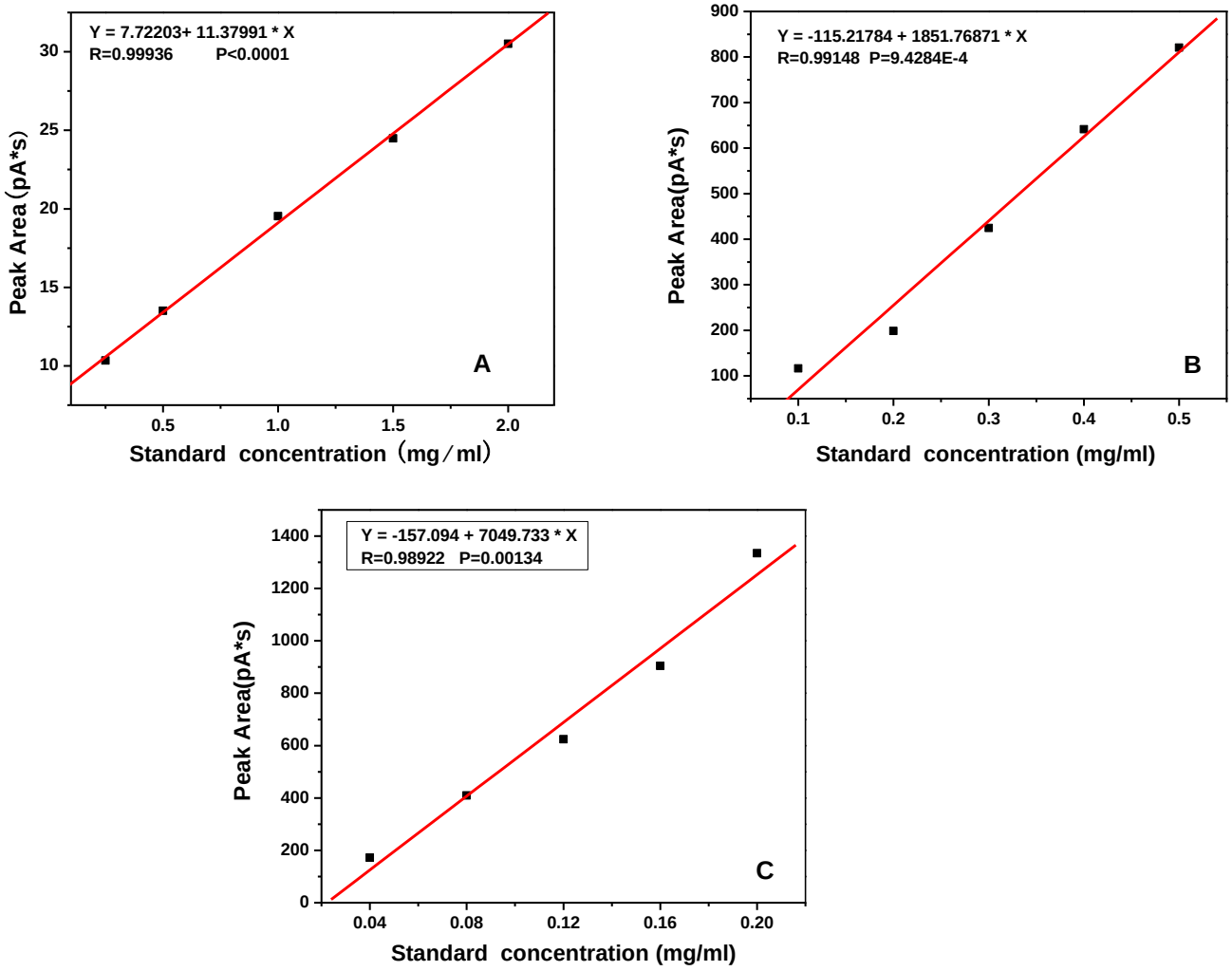

Figure 2. Standard curve (A, spinacene, B, alkyl oxygen; C, vitamin A).

7049.733 * X. Figure 3 show the retention time of spinacene, alkyl oxygen and vitamin A were 11, 15.5 and 6.3 min respectively. The peak area of them were 12.317 pA*s, 538.716 pA*s and 389.483 mAu*s according to the report of chromatographic analysis. According to the standard curve equation, the concentration of spinacene, alkyl oxygen and vitamin A in sample SLO (0.1 mg/ml) were 0.404, 0.353 and 0.078 mg/ml. Thus, the proportion of spinacene, alkyl oxygen and vitamin A in SLO were 40.4, 35.3 and 7.8%, respectively.

Anti-tumor effect of SLO *in vivo*

Tumors in each group were weighted, noted, and the tumor inhibition rate was calculated. The results are shown as Figure 4. The inhibition rate for the high and low dose group is 54.56 and 35.60%, respectively.

Conclusions

In this study, we reported the Extraction and identification of shark liver oil. The best extraction method was alkaline protease method, and the solid-liquid ratio, quality of the enzyme, reaction pH and temperature was 1:2, 2% of the liver quality, 9, 3.5 h and 48°C respectively with the most extraction rate of 30.15%. Characteristic peak of alkoxy glycerol, squalene and vitamin A was presented in the IR spectra of SLO; the proportion of alkoxy glycerol, squalene content and vitamin was 35.3, 40.4 and 7.8%. Anti-tumor effect of SLO is significant with a inhibition rate 54.56%.

Conflict of Interests

The authors declare that they have no any direct financial

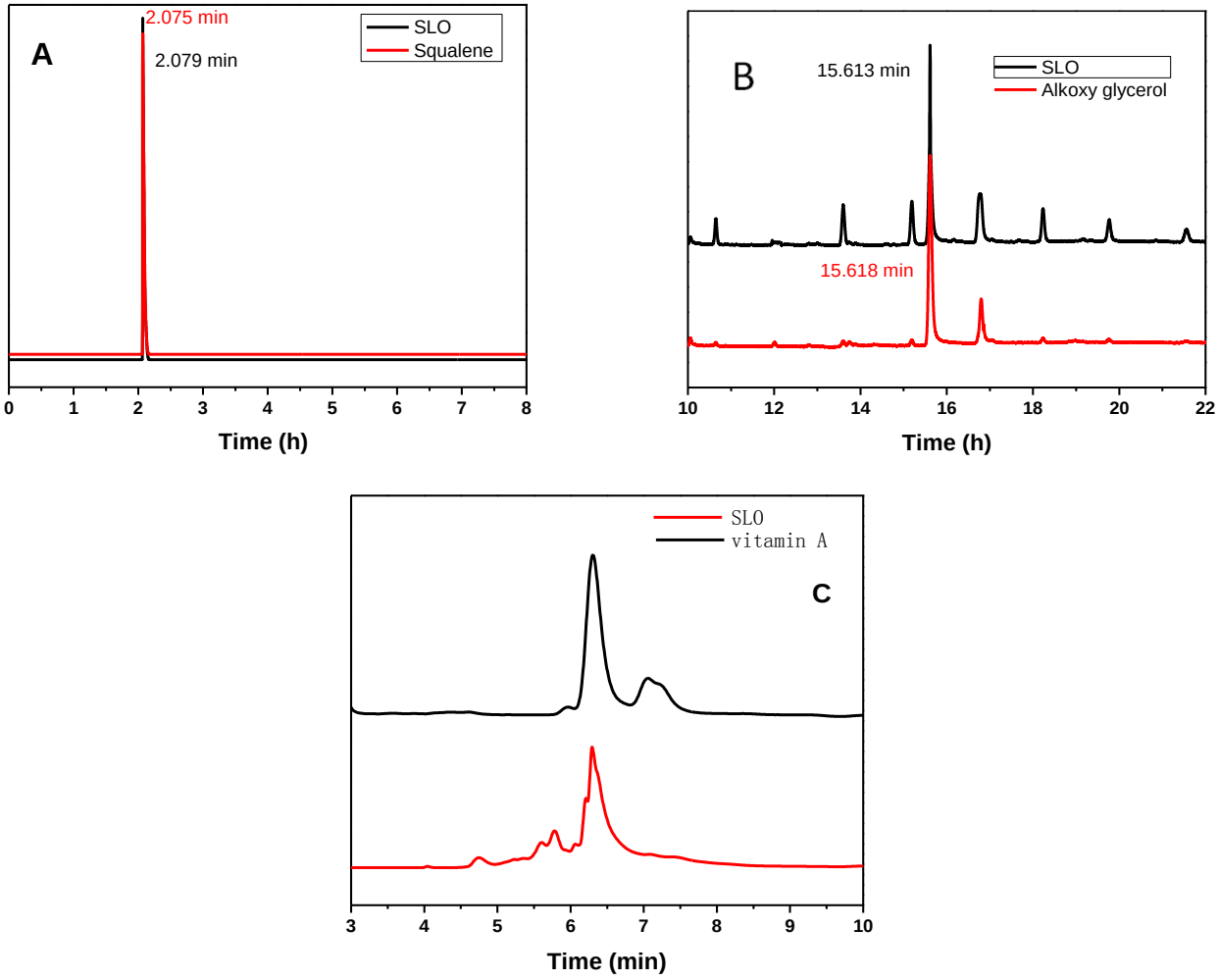

Figure 3. SLO chromatographic analysis (A, spinacene; B, alkyl oxygen; C, vitamin A).

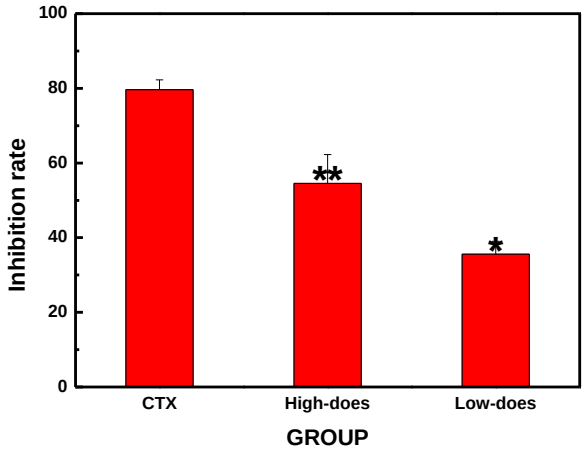

Figure 4. Inhibitive effect of shark liver oil group on tumor inhibition rate. Compared with the negative control group, *p<0.05, **p<0.01; compared with the positive control group, #p<0.05.

relation with the commercial identities mentioned in this paper that might lead to a conflict of interest for any of the authors.

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