ABSTRACT

Introduction: Cynomorium songaricum as a famous Traditional Chinese Medicine (TCM) material, is generally used to cure impotence, premature ejaculation, kidney-yang deficiency, and spermatorrhea. Aim of this research is to study on vitro anti-oxidant and anti-cancer activities of C. songaricum extracts to found its potential in the future medicine and nutrition.

Materials and methods: Polysaccharides, saponins, flavonoids and tannins were extracted from Cynomorium songaricum by Ultrasonic assisted assistant. Anti-oxidant and anti-cancer activity of extracts were analyzed and compared by the method of ultraviolet spectrophotometry and MTT assay respectively.

Results: Conclusion demonstrated that ascorbic acid had the best hydroxyl radical-scavenging assay (scavenging rate was 94.14%), the mitomycin's inhibition rate for cancer cell A549 was 59.31%, and its inhibition rate for cancer cell SMMC-7721 was 53.83%. Tannins had the highest reducing power assay(EC50=0.35) and DPPH radical-scavenging assay (IC50=0.0019) better than ascorbic acid, the highest hydroxyl radical-scavenging assay (46.44%) better than other extracts, and had the good inhibition action for cancer cell A549 (inhibition rate was 41.6%) and SMMC-7721 (inhibition rate was 13.1%). Flavonoids had good reducing power assay (EC50=0.38) and DPPH radical-scavenging assay (IC50=0.0028) which was only next to tannins, and had the best inhibition action for cancer cell A549 (inhibition rate is 45.7%) than other extracts. Saponins had the highest inhibition action for cancer cell A549 (inhibition rate is 36.0%). Polysaccharides have good hydroxyl radical-scavenging rate (39.33%) which was only next to tannins.

Conclusion: Four extracts of C. songaricum showed good anti-oxidant activities and certain anti-tumor activities.

Keywords: Cynomorium songaricum Rupr., Antioxidant activity, Anti-cancer activity.

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Introduction

Increasing evidence shows that the imbalance between production and consumption of reactive oxygen species, leads to oxidative stress and irreversible oxidative damage for many important biological macromolecules, can cause various diseases ultimately, including aging, cancer, neurological degeneration, and arthritis. In addition, the oxygen free radicals are the main cause oxidation for food. Therefore, the potential of antioxidants to prevent oxidation in the body has attracted much attention, and dietary supplements containing antioxidants are available to protect the body from the attack of oxidative stress.

Malignant liver cancer and lung cancer threat to human health seriously. As the primary factor of cancer death most patients is diagnosed in later period of cancer. To explore the drug which has little harm to normal tissues but overkills the tumor
tissues with high accuracy and little side-effect is more important.

*C. songaricum* as an obligate root parasitic plant, usually parasitize on the roots of genera Nitraria (N. tanguticum, N. sibirica, and so on), Zygophyllum xanthoxylon and Peganum harmala. It distributes in the northwest of China and is considered to be a longevity food and good invigorant by the local people. *C. songaricum* as a famous Traditional Chinese Medicine (TCM) material, is generally used to cure impotence, premature ejaculation, kidney-yang deficiency, and spermatorrhea.

The research reports shows that some chemical constituents of *C. songaricum* have antioxidant, anti-diabetes, anti-HIV, anti-aging, improving sexual function and immunity activity, but there is a few study referring to *C. songaricum* polysaccharides and the comparison of extracts’ activities.

In this article, we research on vitro anti-oxidant and anti-cancer activities of *C. songaricum* extracts to found its potential in the future medicine and nutrition when used as drug or food additive.

Materials and methods

**Plant Material**

*C. songaricum* was provided by the Guazhou Yide Biological Technical Co., Ltd. (Gansu Province, China). Supplement of additional washed thoroughly with water and dried at 50°C. The dried *C. songaricum* were then powdered and stored at -4°C until use.

**Cancer cells**

Human lung adenocarcinoma A549 cells and human hepatoma SMMC-7721 cells were obtained from the cell culture room of Lanzhou University. The cancer cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 10% injection of mitomycin at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed two or three times each week.

**Chemicals and Regents**

DPPH (1,1-diphenyl-2-picrylhydrazyl radical) were from Aladdin chemistry Co. Ltd. (Shanghai, China). Some analytical and assay agents, and standards, such as vitamin C, ferric chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate were purchased from Tianjin Baishi Chemical industry Co., Ltd. (Shandong, China). 95% ethanol, n-butanol, ethyl acetate were purchased from Lanzhou Yongda Chemical Co. Ltd. (Gansu, China). Ethanol and 30% hydrogen peroxide were purchased from Tianjin Fuyu chemistry Co.Ltd. (Shandong, China). Salicylic acid was obtained from Tianjin Beicheng Founder chemistry Co. Ltd. (Shandong, China). In addition, we also use the ascorbic acid (Tianjin Chemical Factory, China), AB-8 macroporous resin(Tianjin Nankai University Chemical Factory, China), ferric chloride (Suzhuang, Tianjin, China), potassium ferricyanide (Tianjing Guangfu Fine Chemical Research Institute, China), TCA (Shanghai SSS Reagent Co., Ltd., China), injection of mitomycin (Zhejiang Haizheng Medical Co., China), dimethyl sulfoxide (DMSO, Beijing Biodev, China), fetal bovine serum (sigma-aldrich, USA), RPMI-1640 medium (Gibco Co., Grand Island, NY, USA), MTT and trypsin (Chengpeng Biological Co., China) were used to study antitumor activity. All chemicals were of sufficient purity grades for the required uses.

**Extraction Procedure**

Extraction and isolation of polysaccharides

The 100g solid powder was mixed with 800mL of petroleum ether (1:8 solid/liquid ratio) and extracted 2 times at 65°C water-bath to degrease. The herb residue was isolated by filtrate. The dried herb residue extracted with 80% ethanol extraction temperature 80°C, extracting 2 times (2h for each time) to impurity removal and then dried powder. The powder was mixed with de-ionized (DI) water (1:35 solid/liquid ratio) and ultrasound extraction 2 times in 80°C water-bath at 420w power for 60 minutes. Combined filtrate and then concentrated to about 200mL by evaporation. Free of protein by sevag's way, Ethanol (95% grade) was slowly added at 4:1 volume ratio to the concentrated extract solution, and the mixture was left for 12h at 4°C. The resultant precipitate was collected, and successively washed with anhydrous ethanol and acetone, and then freeze dried, yielding the crude polysaccharides fractions. Finally the sample was purified by AB-8 macroreticular resin. Elution with distilled water and concentrated, dried to obtain cynomorium polysaccharides.

Extraction and isolation of saponins

The 100g Cynomorium powder ultrasound extracted with 65% ethanol extraction temperature 90°C, extracting 2 times (1:15 solid/liquid ratio), at 420w power for 45 minutes. Before it cooling, fil-
The ethanol filtrate was collected and dissolved in a small amount of water and extraction by aether after petroleum ether, one third volume of water extract, each extracting 3 times to degrease. The residual aether will evaporate from the water phase. Adding half water volume n-butyl alcohol to extraction. Concentrated extract solution and make it dissolved in little methanol. Put this methanol solution into a great amount of mixed solvent of ether-acetone (1:1, V/V). The precipitate was collected and vacuum drying in 40°C. Grounded into a fine powder and purified by AB-8 macroreticular resin. Elution with distilled water to impurity and then collect 75% ethanol eluent, concentrated and dried to obtain cynomorium saponins.

**Extraction and isolation of flavonoids**

The 100g solid powder was mixed with 800mL of petroleum ether (1:8 solid/liquid ratio) and extracted 2 times at 65°C water-bath to degrease. The powder extracted with 70% ethanol (1:3 solid/liquid ratio) and ultrasound extraction 2 times in 77°C water-bath at 600w power for 62 minutes. Before it cooling, filtrated and standing overnight. Filtered again to remove the wax, reduced pressure distillation of ethanol and extracted with ethyl acetate 3-5 times. Ethyl acetate was recovered with 2mol/L HCl and extracted with 1/2 volume water. The precipitate was dissolved in little methanol. Put this methanol solution into a great amount of mixed solvent of ether-acetone (1:1, V/V). The precipitate was collected and vacuum drying in 40°C. Grounded into a fine powder and purified by AB-8 macroreticular resin. Elution with distilled water to impurity and then collect 75% ethanol eluent, concentrated and dried to obtain cynomorium flavonoids.

**Extraction and isolation of tannins**

The 100g solid powder was mixed with 1800mL of 75% ethanol (1:18 solid/liquid ratio) and ultrasound extraction 2 times in 80°C water-bath at 600w power for 75 minutes. Before it cooling, filtrated. Combined filtrate and then concentrated to about 200mL by evaporation. After it cooling, the alkaline pH value of solution is adjusted with 2mol/L NaOH, laying it overnight, filtered off precipitate. The acid pH value of solution is adjusted with 2mol/L HCl and extracted with 1/2 volume ethyl acetate several times. Ethyl acetate was recovered under reduced pressure to get extracts. Grounded into a fine powder and purified by AB-8 macroreticular resin. Elution with distilled water to impurity and then collect 75% ethanol eluent, concentrated and dried to obtain cynomorium tannins.

**Measurement of Antioxidant activity**

**Reducing power assay**

Adding 2.5mL of Phosphate buffer (0.2mol/L, pH6.6) in 2.5mL of different concentration of samples, are incubated with potassium ferricyanide (1% W/V) 2.5mL at 50°C for 20 min. The reaction was terminated by adding 2.5mL of TCA solution (10% W/V) and the mixture was centrifuged at 3000r/min for 10min. The supernatant was mixed with 2.5mL of distilled water and 0.5mL of ferric chloride solution (0.1% W/V), stood for 10min and the absorbance was measured at 700 nm. The absorbance of the reaction mixture indicated reducing power and chose ascorbic acid as positive control.

**DPPH radical-scavenging assay**

Solution of DPPH in ethanol (0.025mg·mL-1) and mixed with 2mL of the samples which diluted with ethanol at different concentrations. The mixture in the test tubes was shaken for 30 min at room temperature, and then measured absorbance at 515 nm. Ascorbic acid was used as positive controls, 2mL of ethanol always measured as control group, and the antioxidant capacity of samples was calculated as follows:

\[
\text{Scavenging (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{black}}}\right] \times 100%.
\]

where \(A_{\text{sample}}\) = absorbance of test, \(A_{\text{black}}\) = absorbance of blank (2mL of ethanol instead of sample). \(A_{\text{control}}\) = absorbance of control (2mL of ethanol instead of sample).

IC50 value was determined to be the effective concentration at which DPPH radical was scavenged by 50%. IC50 more low means the scavenging activities of the samples more high. The IC50 value was obtained by interpolation from linear regression analysis.

**Hydroxyl radical-scavenging assay**

Added 6mmol/L ferrous sulfate solution 1mL, Salicylic acid-ethanol mixes 1mL, different concentration of sample 1mL, 0.1% Hydrogen Peroxide 1 mL into a 10mL of colorimetric tube and constant volume. Water bath heat for 60 min in constant temperature(37°C), and then measured absorbance at 510 nm. Ascorbic acid was used as positive controls, and the antioxidant capacity of samples was calculated as follows:

\[
\text{Scavenging (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{black}}}\right] \times 100%.
\]
where $A_{\text{sample}}$ = absorbance of test (including ferrous sulfate solution, Salicylic acid-ethanol mixtures, Hydrogen Peroxide, sample), $A_{\text{black}}$ = absorbance of blank (including ferrous sulfate solution, Salicylic acid-ethanol mixtures, Hydrogen Peroxide), $A_{\text{control}}$ = absorbance of control (including ferrous sulfate solution, Salicylic acid-ethanol mixtures, sample).

**Measurement of anti-cancer activity**

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltriazolium bromide (MTT) assay. The anti-cancer effects of polysaccharides, saponins, flavonoids and tannins were assessed with an MTT assay. The cells were seeded in a 96-well plate ($1 \times 10^5$ cells/mL per well) in a volume of 90 µL. Subsequently, 10 µL samples were added. The cells were subsequently incubated with the samples solutions for 48 h at 37°C in an incubator in a humidified atmosphere containing 5% CO$_2$. MTT solution (20µL, 5mg/mL) was added to each well and the cells were cultured for a further 4h under the same conditions. Following the removal of the supernatant, 100 µL DMSO was added to each well and oscillated for 10 min. Finally, the absorbance of each well was measured using an ELISA plate reader at 490 nm.

**Results and discussion**

**Results of antioxidant activity**

**Results of reducing power assay**

Experimental data (as shown in Fig.1) explain that there was a dose-effect relationship between concentration range and reducing power, i.e. with the increasing of the concentration, reducing power increased. Saponins’ absorbance value (Abs) was 0.5474, higher than ascorbic acid (Abs is 0.5053) when samples’ concentration at 0.06mg/mL in contrast with 0.1mg/mL. When the samples’ concentration at 0.1mg/mL, the sequence of reducing power about four extracts were tannins (Abs=1.1790) > flavonoids (Abs=1.0030) > ascorbic acid (Abs=0.9263) > saponins (Abs=0.8105) > polysaccharide (Abs=0.4421).

The half maximal effective concentration ($EC_{50}$) shows in Table1 that the saponins, flavonoids and tannins of *C. songaricum* have strong reducing ability and the average concentration of EC50 was less than ascorbic acid. The tannins and flavonoids extracts exhibited strong radical scavenging activity because there had similar EC50 about 0.3mg/mL. The saponins’ EC50 was close to ascorbic acid about 0.5mg/mL. The polysaccharides’ EC50 was 0.1239mg/mL that higher two times than ascorbic acid. The sequence of reducing power about four extracts were tannins > flavonoids > saponins > ascorbic acid > polysaccharide. The tannins and flavonoids extracts exhibited strong radical scavenging activity.

**Results of DPPH radical-scavenging assay**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration range[mg/mL]</th>
<th>linear relationship</th>
<th>EC50[mg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ascorbic acid</td>
<td>0.02–0.1</td>
<td>$y = 9.9100 x - 0.0382$ ($R^2 = 0.9926$)</td>
<td>0.0543</td>
</tr>
<tr>
<td>polysaccharides</td>
<td>0.025–0.25</td>
<td>$y = 3.6079 x+ 0.0530$ ($R^2 = 0.9936$)</td>
<td>0.1239</td>
</tr>
<tr>
<td>saponins</td>
<td>0.02–0.1</td>
<td>$y = 7.3300 x + 0.1180$ ($R^2 = 0.9802$)</td>
<td>0.0521</td>
</tr>
<tr>
<td>flavonoids</td>
<td>0.02–0.1</td>
<td>$y = 9.0100 x + 0.1524$ ($R^2 = 0.9862$)</td>
<td>0.0386</td>
</tr>
<tr>
<td>tannins</td>
<td>0.02–0.1</td>
<td>$y = 9.9350 x + 0.1459$ ($R^2 = 0.9903$)</td>
<td>0.0356</td>
</tr>
</tbody>
</table>

Table 1: the comparison of EC50 in reducing power between four extracts of *C. songaricum* and ascorbic acid.

Note: EC50 (Concentration for 50% of maximal effect) was the sample concentration when absorbance was 0.5.

Figure 2 show that there was a dose-effect relationship between concentration range and DPPH radical-scavenging rate, with the increasing of the concentration, DPPH radical-scavenging rate increased. Concentration of sample had linear relation with scavenging rate in some concentration range. When the samples’ concentration less than 0.5mg/mL, tannins always had the highest scavenging rate, saponins and flavonoids were similar with ascorbic acid.

The scavenging rate more than 90%, the rising trend of scavenging rate became very slow when
concentration exceeded 0.008mg/mL except polysaccharides which always had the lowest scavenging rate. Four extracts of C. songaricum and ascorbic acid had linear relation with DPPH radical-scavenging rate show in table 2.

### Table 2: the comparison of IC50 in DPPH radical-scavenging rate between four extracts of C. songaricum and ascorbic acid.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration range[mg/mL]</th>
<th>linear relationship</th>
<th>IC50[mg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ascorbic acid</td>
<td>0.001~0.008</td>
<td>$y=85.8791 x + 0.2324$ ($R^2 = 0.9704$)</td>
<td>0.0031</td>
</tr>
<tr>
<td>polysaccharides</td>
<td>0.002~0.0016</td>
<td>$y=46.8122 x + 0.0951$ ($R^2 = 0.9660$)</td>
<td>0.0086</td>
</tr>
<tr>
<td>saponins</td>
<td>0.001~0.008</td>
<td>$y=86.7539 x + 0.2196$ ($R^2 = 0.9623$)</td>
<td>0.0032</td>
</tr>
<tr>
<td>flavonoids</td>
<td>0.001~0.008</td>
<td>$y=84.2035 x + 0.2637$ ($R^2 = 0.9665$)</td>
<td>0.0028</td>
</tr>
<tr>
<td>tannins</td>
<td>0.0005~0.0004</td>
<td>$y=176.5670x+0.1729$ ($R^2 = 0.9703$)</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

Note: IC50 (50% inhibitory concentration of a substance) was the sample concentration when DPPH radical-scavenging rate was 50%.

50% inhibitory concentration of a substance (IC50) shows that the tannins of C. songaricum have a good inhibitory action on DPPH•, its IC50 was 0.0019mg/mL. The flavonoids and saponins were close to ascorbic acid. The polysaccharides had the highest IC50, so had the lowest DPPH radical-scavenging rate. The sequence of DPPH radical-scavenging rate were tannins > flavonoids > ascorbic acid > saponins > polysaccharide. DPPH radical-scavenging rate of saponins is very similar to ascorbic acid.

### Results of hydroxyl radical-scavenging assay

Figure 3 show that there was a dose-effect relationship between concentration range and hydroxyl radical-scavenging rate in some concentration range. When the concentration of sample rises, the hydroxyl radical-scavenging rate rises. The comparison of hydroxyl radical-scavenging rate between four extracts of C. songaricum and ascorbic acid, ascorbic acid had a better ability than other extracts. Tannins had the best scavenging no matter in which concentration. Saponins were similar with flavonoids and lower than ascorbic acid slightly. Polysaccharides’ scavenging rate (22.18%) lower than saponin ((27.62%) and flavonoids (31.80%) in 0.4mg/mL of concentration, but had exceeding them in 0.5mg/mL. When the samples’ concentration at 0.5mg/mL, ascorbic acid radical-scavenging rate was 94.14%, the tannins was 46.44%, and the saponins had the lowest rate that was 32.22%. The sequence of radical-scavenging ability about four extracts were ascorbic acid > tannins > polysaccharide > flavonoids > saponins.

### Results of anti-cancer activity

The antitumor activity of four extracts was measured by using MTT assay. The results of his experiment shows, when the sample concentration was 100μg/mL, the most inhibition ability of cancer cell A549 was flavonoids, the inhibition rate of cancer cell is 45.68%. The tannins was close to flavonoids. The polysaccharide has the lowest inhibition rate about 33.27%. The saponins were a little higher than polysaccharide. The sequence of anti-cancer activity about four extracts on cancer cell A549 were mitomycin > flavonoids > tannins > saponins > polysaccharide. There is a few inhibition action for cancer cell SMMC-7721 except saponins which inhibition rate was 24.98%. The tannins had 13.08% inhibition rate of cancer cell. Especially, flavonoids on SMMC-7721 showed growth promotion effect.

### Conclusion

The reducing power activities, DPPH radical-scavenging activities, Hydroxyl radical -scavenging
activities, anti-cancer activities and its extracts of *C. songaricum* were evaluated in vitro. Result of reducing power was similar to DPPH radical-scavenging rate, the sequence of antioxidant activity about four extracts were tannins > flavonoids > saponins > polysaccharide, and saponins is very similar to ascorbic acid. Result of hydroxyl radical-scavenging rate shows that the sequence of antioxidant activity about four extracts were tannins > polysaccharide> flavonoids > saponins, and those entire compound were lower than ascorbic acid.

Conclusion demonstrated that tannins have the best antioxidant activity. The sequence of anti-cancer activity about four extracts on cancer cell A549 were mitomycin > flavonoids > tannins > saponins > polysaccharide.

The most inhibition ability of cancer cell A549 was flavonoids; polysaccharide has the lowest inhibition rate. As a result, four extracts of *C. songaricum* exhibited higher antioxidant activities, and displayed higher antitumor activity on cancer cell A549. However, they did not significantly delay SMMC-7721 cells growth. There were only two tumor cells were used in anti-tumor assay, which cannot adequately demonstrate the *C. songaricum* have the antitumor effects. To have a better understand about *C. songaricum*, it is suggested using more strains of multi-tumor cell in the further research.

The results show that *C. songaricum* extracts are rich in antioxidant and anti-tumor components, but the composition and the presence form of antioxidants may be quite different, so different extracts have different antioxidant and anti-tumor. Thus this article can give us a reference in *C. songaricum* development and utilization.

Ascorbic acid had the best hydroxyl radical-scavenging rate (94.14%), the mitomycin’s inhibition rate for cancer cell A549 was 59.31%, and its inhibition rate for cancer cell SMMC-7721 was 53.83%. Tannins had the highest reducing power assay (EC₅₀=0.35) and DPPH radical-scavenging rate(IC₅₀=0.0019) better than ascorbic acid, the highest hydroxyl radical-scavenging rate (46.44%) better than other extract, and had the good inhibition action for cancer cell A549 (inhibition rate was 41.6%) and SMMC-7721(inhibition rate was 13.1%). Flavonoids had good reducing power assay (EC₅₀=0.38) and DPPH radical-scavenging assay (IC₅₀=0.0028) which was only next to tannins, and had the best inhibition action for cancer cell A549 (inhibition rate is 45.7%) than other extracts.

Saponins have the highest inhibition action for cancer cell A549 (inhibition rate is 36.0%) better than others, and other activities were ordinary. Polysaccharides had good hydroxyl radical-scavenging rate (39.33%) which was only next to tannins, and other activities are not outstanding. Four extracts of *C. songaricum* showed higher anti-oxidant activities and certain anti-tumor activities. They had a huge potential in drug discovering and developing if we want to obtain drugs whose have good activities such as high total reducing power, inhibition rate and so on. It can guide us to make good use of *C. songaricum*.

The results indicate that *C. songaricum* could be a potential source of natural antioxidants. Therefore, the results provide useful information of *C. songaricum* on pharmacological activities and potential applications of such extracts as natural antioxidants in different foods/pharmaceutical products.

### References


<table>
<thead>
<tr>
<th>Sample[100µg/mL]</th>
<th>inhibition rate of cancer cell A549 (%)</th>
<th>inhibition rate of cancer cell SMMC-7721 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitomycin for injection</td>
<td>59.31±0.8810</td>
<td>53.8290±1.9825</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>33.26±7.14600</td>
<td>7.8057±2.7889</td>
</tr>
<tr>
<td>Saponins</td>
<td>35.96±33.1.0361</td>
<td>24.98±41.11.8223</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>45.68±33.1.9995</td>
<td>-1.04±09±1.5613</td>
</tr>
<tr>
<td>Tannins</td>
<td>41.5±63±4.2184</td>
<td>13.8055±1.0541</td>
</tr>
</tbody>
</table>

**Table 3**: the inhibition of cancer cell A549 and SMMC-7721 by four extracts of *C. songaricum*.


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