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Antiangiogenic Activity of Glycyrrhiza Extract

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Abstract

The bioactivity of extract of glycyrrhiza was detected by zebrafish antiangiogenic model after 70% ethanol extract of glycyrrhiza was extracted with petroleum ether, ethyl acetate, n-butanol. The inhibition of the extracts in antiangiogenic activity showed that the highest active components existed in ethyl acetate extract of glycyrrhiza. The ethyl acetate extract of glycyrrhiza was separated by polyamide column chromatography to obtain 7 fractions (Fr1-Fr7), which Fr5 and Fr6 showed antiangiogenic activity.

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Keywords- antiangiogenic activity; glycyrrhiza extract; zebrafish antiangiogenic model

1. Introduction

The tumor is one of universally accepted diseases that could do severe harm to people. The morbidity of tumor is taking on the rising trend due to effects of bad environment and other adverse factors. Angiogenesis of tumor is an essential condition for its rapid increase because of supplying nutrition for growth and metastasis of tumor cells. Preventing to generate tumor vessel net by restraining angiogenesis of tumor could reduce the growth and metastasis of tumor cells. Compared with conventional chemotherapy drugs, antiangiogenic agents have several advantages, such as definite target, low toxicity, no drug resistance. Thus research and exploitation of the antineoplastic agents become a hot spot of drugs research now[1-5].

Zebrafish antitumor drug model is an established drug screening model, which has some distinguished advantages as a screening model for antiangiogenesis antitumor drugs such as simply operation and high throughput screening. There is no seasonality for zebrafish reproduction. One female zebrafish could produce hundreds of ovums every week, providing plentiful embryo for high-throughput pharmaceuticals screening. The embryonic development of zebrafish need so short screening cycle time to be formed for 24-72 h. The embryo of zebrafish is transparent and easy to observe angiogenesis in vivo. The embryo is very small and need little amount of drugs (μg) in experiment. Measured compounds is easy to dissolve in water and diffuse into embryo or be injected into embryo directly. Zebrafish belongs to vertebrates and has the similarity of 85% to human in central nervous system, internal organs, blood and vision system. So the ingredients screened by zebrafish model have high relevance to human. The cost of
feeding and reproduction of zebrafish is far low than other experimental animal [6-8]. The screening bioactive compounds is novel pharmaceutical agents’ beginnings, research and development of new pharmaceutical agents can not achieve success without screening, so advanced drug screening model could improve the speed and success rate of new drugs discovery and low the cost of research and development [9-11].

Our laboratory did a lot work in screening Chinese medicinal plant by zebrafish anti-angiogenesis screening model, initial discovery of the anti-angiogenesis activity of glycyrrhiza crude extract. Chemical composition of glycyrrhiza were separated by HSCCC, and active ingredients were tracked with the help of zebrafish anti-angiogenesis screening.

2. Materials and instruments

2.1 Experimental materials

Glycyrrhiza (bought from local herb stores), petroleum ether, acetic ether, n-butanol, Ethanol, Dimethyl sulfoxide (analytical pure), anesthetic Tricaine (Sigma company), polyamide resin (80-100 mesh, 4A biochemistry plastic factory from taizhou city, Zhejiang province), GF254 silicon board(50×100mm, Puke separation materials from Qingdao Co. Ltd)

2.2 Instruments and equipments

Ultraviolet transilluminator reflectometry (Kanghua biochemical instrument manufactory from Shanghai). XSJ-D inverted biological microscope (Chongqing optical instrument factory). Illuminating incubator (Shanghai Jixing biotechnology Co. Ltd.)

3. Methods

3.1 Extraction and separation active ingredients from glycyrrhiza

Glycyrrhiza 1 kg was dried, crushed to 80 mesh, heating reflux with 6 times volume ethanol (70%), the treatment was repeated 3 times, then released pressure for recycling alcohol. The extract was dissolved in 3 L hot water, and extracted with same volume petroleum ether, ethyl acetate, n-butanol in turn, and each extraction process repeated 5 times. After reduced pressure distillation, we got petroleum ether extract 4g, ethyl acetate extract 30 g, n-butanol extract 12 g, water extract 53 g. Then those extracts activity were detected with zebrafish anti-angiogenesis drugs screening model, and the higher anti-angiogenesis bioactivity of the ethyl acetate extracts was discovered.

Use the polyamide resin column for ethyl acetate extracts’ further separation. Specific operation is: ethyl acetate extract was dissolved in some alcohol, added 2 times weight polyamide resin, stirred into homogenized and then on the pretreated polyamide (300g) column (5 cm×100 cm) gel surface, gradient eluted with water, 30% alcohol, 50% alcohol, 70% alcohol, 95% alcohol in turn, each gradient washed with 5 times volume elution, current velocity at 0.3 BV/h, 500mL as a fraction. After collected fractions concentrate, the same fractions were combined according to silicon thin layer chromatography results, we got 7 fractions: Fr1 (1.82g), Fr2 (2.44 g), Fr3 (5.44 g), Fr4 (4.16 g), Fr5 (6.25 g), Fr6 (3.47 g), Fr7 (2.36 g). Fr1~Fr7 fractions were detected activity and determine their main active parts by zebrafish anti-angiogenesis drugs screening model. Flow Chart as shown below:
3.2 Preparation for standard curve

Samples 10 mg were transferred to a 1.5 mL centrifuge tube and 100 μL dimethyl sulfoxide (DMSO) were added. After the mixture was homogenized, we obtained 100mg/mL sample solution. Mother liquor was diluted with DMSO, obtained 10 mg/mL, 1 mg/mL mother liquor and was used for embryo culture later. Three kinds of concentration solution mentioned above were diluted for 100 times and obtained 1000 μg/mL, 100 μg/mL, 10 μg/mL sample solution, for later use.

3.3 Preparation for embryos

Experimental zebrafishes are AB strains, and its cultivation method as described by Westerfield. Chosen sexually mature one male and one female zebrafish and put them all in tank for fertilization later in the afternoon, fertilized eggs were collected in the next morning 6–8 o’clock. Fertilized eggs were transferred by pipette (point was cut off) to 0.5% sodium hypochlorite solution (prepared with embryo culture water), and then was shaken lightly to wash and sterilize embryos, transferred into embryo culture water after 2 min, then put it in a temperature control (28°C) illuminating incubator culture 24 h, for later use. When the development of zebrafish embryos reach to 24 h, chorionic villus of zebrafish embryos were digested and dissolved by 1mg/mL trypsin (prepared when needed). Ten minutes later, chorionic villus began fall off, and then wash with large amounts of water, repeated 5 times, obtained zebrafish embryos without any chorionic villus.

3.4 Treatment embryos with glycyrrhiza extract solution

We chosen 96-well plates and each well added with 30 μL sample solution, 8 wells in a row were filled with the same sample and same concentration solution. Each well was added with single no chorionic embryo, and then full with water (about 300 μL), that was the process of pesticide, and each sample got 3 kinds of concentration 1, 10, 100 μg/mL, and set a blank control group (embryo culture water with 1% DMSO). Cover the 96-well plates with a lid, put it in temperature control (28°C) illuminating incubator culture and for further culture. We observed the results at 48 hours (embryonic development 72h) after medication. Remove 30 μL water from each well of 96-well plates, then added with 30μL 1‰ anesthetic (Tricaine) to got anesthetized fish to facilitate the observation. After that, transferred the fish to a microscopic slide with a pipette (point was cut off), observed under XSJ-D inverted biological microscope, counted the zebrafish intersegmental vessels.

3.5 Statistical analysis

Calculate angiogenesis inhibition ratio as follow:
Inhibition ratio = (Vessel numbers of control group – Vessel numbers of medicine group) / Vessel numbers of control group × 100%

Data are presented as means and standard deviations. SPSS (v17.0) was used for statistical analyses.

4 Results and discussion

4.1 Different solvent influences on Glycyrrhiza extracts anti-angiogenesis activity

When the embryos of zebrafish development reach to 24 h, added with different concentrations different fractions glycyrrhiza extracts, cultured at temperature control (28°C) illuminating incubator, chosen embryonic development 72 h, observed under XSJ-D inverted biological microscope, counted the
zebrafish intersegmental vessels. Zebrafish embryos almost all were dead with the different fractions glycyrrhiza extracts concentration 100 μg/mL for its high concentration; Zebrafish embryos normally grew and show no differences obviously with control group when the concentration is 1 μg/mL; But when the concentration is 10 μg/mL, there are great differences between sample groups, as the figure 4.1 shown: glycyrrhiza ethyl acetate extract has obvious antiangiogenic activity, appeared significantly different with the control group, and its vascular inhibition ratio reach to 30%, while there were no difference in control group and other fractions extracts group.

4.2 Angiogenesis activity of eluted fractions through polyamide resin column

After screening for glycyrrhiza four kinds of extracts through zebrafish anti-angiogenesis drugs screening model, we discovered ethyl acetate extract higher antiangiogenic activity. Further separation with polyamide resin column, ethyl acetate extracts were divided into 7 parts (Fr1-Fr7) according to the results of silica gel thin-layer chromatography (Figure 4.2). Activity of these 7 fractions sample were determined by using zebrafish anti-angiogenesis drugs screening model, and when the sample concentration is 100 μg/mL, zebrafish embryos grow well in Fr1-Fr3 sample groups, no capillaries (smallest vessels) absence and the mortality of zebrafish embryos in Fr4-Fr7 sample groups is very high. When the sample concentration is 1 μg/mL, there are no vessels deficient phenomenon and zebrafish embryos all are grow well except individual embryo from Fr5, Fr6 sample groups. When the sample concentration is 10 μg/mL, fractions Fr4-Fr7 have antiangiogenic activity, and Fr4, Fr7 only have weak activity, inhibition ratio reached to about 20%; Fr5, Fr6 fractions have higher activity and capillaries inhibition ratio reached to 64% and 76%, respectively.

5. Conclusions

In recent years, the number of malignant tumor patient was showed an upward trend year after year. Chemotherapy as an important means to cure malignant tumor but significant toxicity and side effects also were accepted by doctors. So, the research and development of antitumor drugs of accurate target, minimal side effect, and better effect has important social and economy benefits. Inhibiting angiogenesis antitumor agents having those advantages was founded in recent years and new drugs development must been supported by corresponding animal screening model. At present, the main model for angiogenesis qualitative and quantity study in vivo are chicken chorioallantoic membrane (CAM) angiogenesis, mouse embryonic angiogenesis, rabbit auricular and animal transplant under skin angiogenesis, pial mater and mesentery angiogenesis, etc. Zebrafish embryo model has high throughput screening, short screening cycle time, high degree of screening accuracy virtues compare to those models above.

In this paper, we studied glycyrrhiza active ingredients tracking and separation by zebrafish anti-angiogenesis drugs screening model, determined the active points of glycyrrhiza. First, ethyl acetate glycyrrhiza extract anti-angiogenic activity was identified by using the zebrafish anti-angiogenesis drugs screening model, but other glycyrrhiza crude fractions petroleum ether, ethyl acetate, n-butanol and water extract didn’t. Then, the ethyl acetate extracts were separated into seven fractions (Fr1-Fr7) with polyamide resin column and Fr5, Fr6 showed highest antiangiogenic activity after further screening of those 7 fractions. The scope of active compounds exists was narrowed through screening, prepared the ground for active monomers’ further separation and purification.

References


Figure 4.1 Antiangiogenic activity of different extracts of Glycyrrhiza. 1, 2, 3, 4, and 5 stand for control, petroleum ether, ethyl acetate, n-butanol and water (**, P<0.01.)
Figure 4.2 TLC analyses of the fractions from polyamide column chromatography separation.