

# Assessment of the embryotoxicity of four Chinese herbal extracts using the embryonic stem cell test

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**Abstract.** Rhizoma *Atractylodes macrocephala*, *Radix Isatidis*, *Coptis chinensis* and *Flos Genkwa* are common herbal remedies used by pregnant woman in China. In this study, their potential embryotoxicity was assessed using the embryonic stem cell test (EST) and a prediction model. The potential embryotoxicity of the herbs was based on three endpoints: the concentrations of the compounds that inhibited the proliferation of 50% of embryonic stem cells (ESCs) (IC<sub>50</sub>ES), the concentrations that inhibited 50% of 3T3 cells (IC<sub>50</sub>3T3), and the concentrations that inhibited the differentiation of 50% of ESCs (ID<sub>50</sub>ES). The results revealed that Rhizoma *Atractylodes macrocephala* and *Radix Isatidis* are non-embryotoxic compounds. *Coptis chinensis* extracts appeared to demonstrated weak embryotoxicity, and *Flos Genkwa* exhibited strong embryotoxicity. These results may be useful in guiding the clinical use of these herbs and in expanding the application of the EST to the field of traditional Chinese medicine.

## Introduction

Chinese medicine has played an invaluable role in the prevention and treatment of diseases for thousands of years in China. Although Chinese medicine was considered to have less toxicity and fewer side effects than traditional medicine, increasing evidence has shown this to be untrue (1-3). Given that many pregnant women use Chinese herbs for pregnancy-related

sickness (4,5), the potential developmental toxicity of these herbs needs to be studied. In the present study, we investigated the safety and possible embryonic toxicity of four common herbs: Rhizoma *Atractylodes macrocephala*, *Radix Isatidis*, *Coptis chinensis* and *Flos Genkwa*.

*Atractylodes macrocephala* has been used in traditional Chinese medicine (TCM) for approximately 2,000 years. It exhibits multiparmacological effects and is used to treat various complaints, including excessive vaginal bleeding (6). It is also used to invigorate the spleen (7). As a typical Chinese herbal medicine for replenishing qi, Rhizoma *Atractylodes macrocephala* has long been used in the treatment of threatened preterm labor.

*Radix Isatidis* is the dried root of crucifer *Isatis tinctoria* L, which is widely distributed in northern and central China. According to a book on herbal medicine written in 110 B.C. by Shennong, a notable ancient Chinese medicinal specialist, it has been used as a medicinal plant for more than 2,000 years. It is used to dissipate heat (cold compress), detoxify the immune system and cool the blood. It is widely used for preventing and treating infectious diseases caused by viruses, including influenza, viral pneumonia, mumps and hepatitis (8).

The use of *Coptis chinensis* as a herbal remedy was also first recorded in Shennong's book. This herb is used to dissipate heat and promote diuresis. It is used as a common herbal medicine for diarrhea, dysentery, acute febrile and suppurative infections and vomiting, as well as to protect the gastric mucosa (9). *Coptis chinensis* has been used for thousands of years in China. However, it has been banned in Singapore since 1978 due to the belief that taking this herb during pregnancy or lactation causes serious jaundice in infants.

*Flos Genkwa*, the dried flower buds of *Daphne genkwa*, is a medicinal plant distributed mainly in mainland China. It is commonly used as an abortifacient (10), with purgative, diuretic and anti-inflammatory actions (11). Since the enactment of China's one-child family planning policy, studies of *Flos Genkwa* have focused on its abortion efficacy and mechanisms (12). One previous study has investigated its anticancer actions (13).

A systematic study of the use of Chinese herbal medicines during pregnancy has not been conducted. Currently, teratogenicity tests *in vivo* or genetic toxicity tests are the main methods

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employed to study the reproductive and developmental toxicity of TCM (14,15). Whole embryo cultures or micromass embryo cell cultures have also been used to reveal the developmental toxicity of TCM (16). Comparatively speaking, *in vivo* tests, based on maternal or embryonic exposure of laboratory animals, are more time-consuming and expensive. *In vitro* assays, including the whole-embryo culture assay and the micromass assay, offer an alternative to *in vivo* assessment, although both still rely on embryos (17). Spielmann and Liebsch developed the embryonic stem cell test (EST), an *in vitro* assay system to determine the teratogenic potential of test chemicals (18). It is the only test not requiring pregnant animals (19). The EST is based on murine-derived embryonic stem cells (ESCs) from the blastocyst stage. ESCs are pluripotent cells. They differentiate *in vitro* into a wide variety of cell types, representing all three germ layers (ectoderm, mesoderm and endoderm). Under appropriate culture conditions, certain ESCs differentiate spontaneously into beating myocard cells. Three different endpoints are evaluated in the EST: The inhibition of growth (cytotoxicity) of 3T3 cells and ESCs after 10 days of treatment, determined by the Cell Counting Kit-8 (CCK8) cell proliferation assay, and the inhibition of the differentiation of ESCs into myoblasts following 10 days of treatment. The concentration  $\pm$  response correlations are recorded, and 50% inhibition concentrations are determined for the three endpoints. In the EST, the mutagenic potential of the test substances are classified into three different classes of *in vivo* embryotoxic potencies: strongly embryotoxic, weakly embryotoxic and non-embryotoxic (20). In a previous study, the EST was used to verify the embryotoxicity of 20 reference compounds with the different embryotoxic potencies mentioned above. The accuracy of the EST assay was 78%. Notably, a predictivity of 100% was attained for strong embryotoxicants (21). As a result, the validated EST has been accepted for assessing the embryotoxicity of test compounds at an early stage of development (22). However, there are few reports on the application of the EST to the study of the reproductive and developmental toxicity of TCM (23,24).

The aim of the present study was to evaluate the embryonic developmental toxicity of extracts of four TCMs (*Rhizoma Atractylodes macrocephala*, *Radix Isatidis*, *Coptis chinensis* and *Flos Genkwa*). For this purpose, a modified EST was used. Three endpoints ( $IC_{50}3T3$ ,  $IC_{50}ES$  and  $ID_{50}ES$ ) were used for each extract, and each test compound was classified as strongly, weakly or non-embryotoxic.

## Materials and methods

**Preparation of crude drug extracts.** Roots of *Atractylodes macrocephala*, *Coptis chinensis*, *Radix Isatidis* and *Flos Genkwa* were purchased from Caizhiling, a reputable Chinese medicinal herb store in Guangzhou, China. Their authenticity was confirmed by Professor Ma Zhiguo of the Pharmacy College of Jinan University. The aqueous extract was prepared by a general method. After cutting the herbs into small pieces, 100 g dried plant material was boiled in 1,000 ml distilled water for 1 h. The decoction was collected and the residue was boiled another two times. The decoction obtained from the three separated extractions was mixed, filtered and lyophilized by freeze drying. The powdered forms of the extracts were stored at  $-20^{\circ}C$ .

**Preparation of extracts and pure compound solutions.** The dried extracts were dissolved in double-distilled water until the initial concentration was 1 g/ml and centrifuged at 14,000 g for 5 min before filtration sterilization to obtain a clear, sterile supernatant for testing. The chemicals were dissolved in appropriate solvents. The chemical 5-fluorouracil (5-FU; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulphoxide. Phenytoinum natricum (DPH; Sigma-Aldrich) and saccharin (SAC; Sigma-Aldrich) were dissolved in phosphate-buffered saline (PBS). The final solvent concentrations applied in the differentiation and cytotoxicity assays demonstrated no undesired background effects.

**Cell culture.** Undifferentiated mouse ESCs of the OG<sub>2</sub> cell line were purchased from the Chinese Academy of Sciences. Continuous cultures of the cell line were grown on mitomycin C inactivated mouse embryonic fibroblast (MEF) feeders in a standard culture medium consisting of 80% high-glucose (4.5 g glucose/l) Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Karlsruhe, Germany), 15% fetal calf serum (Hyclone, Erembodegem-Aalst, Belgium), as well as 2 mM glutamine, 2 mM sodium pyruvate, antibiotics (50 U/ml penicillin and 50  $\mu$ g/ml streptomycin), 1% non-essential amino acids, 0.1 mM  $\beta$ -mercaptoethanol and 1,000 U/ml murine leukemia inhibitory factor, which were all purchased from Gibco Life Technologies. BALB/c 3T3 fibroblasts, purchased from the cell bank of Sun Yat-sen University of Medical Sciences, were cultured in DMEM supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50  $\mu$ g/ml streptomycin. The cells were maintained under 5% CO<sub>2</sub> and 95% humidity at 37°C.

**Alkaline phosphatase staining.** The ESCs were subjected to alkaline phosphatase (AKP) staining on day 5 of passage. The original medium was discarded from the culture plates, and the cells were washed with PBS and fixed in 4% para-formaldehyde for 10 min. They were then washed again with PBS and stained with 75 mg/ml nitroterazolum blue chloride (Inogent, Hyderabad, India) and 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Innogent) for 1 h at room temperature.

**Cytotoxicity assay.** The cytotoxic effects on the mESCs and 3T3 cells were detected by performing a CCK8 (Mbchem, Mumbai, India) cell proliferation assay on day 10. In brief, on day 1, 500 cells were seeded into each well of a 96-well tissue plate in a routine cell culture medium without leukemia inhibitory factor and incubated for 2 h. Following incubation, 200  $\mu$ l culture medium containing the appropriate dilution of extracts from the four medicines were added to each well. On days 3 and 5, the medium was renewed. On day 10, CCK8 assay was carried out. Cytotoxicity was expressed as the concentration of the compound that reduced the viability of cells to 50% of the control level ( $IC_{50}3T3$  and  $IC_{50}ES$ ), determined from a concentration-response curve.

**Differentiation assay.** Differentiation assays were performed to detect compound-induced changes in the differentiation of the mESCs into contracting cardiomyocytes. Briefly, on day 0, 20  $\mu$ l stem cell suspension containing 750 cells was placed as

hanging drops on the inner side of the lid of a petri dish filled with 6 ml PBS, then incubated for three days at 37°C, with 5% CO<sub>2</sub> and 95% humidity, in the presence of the test chemicals at various concentrations. During this period, the cells formed aggregates referred to as embryonic bodies (EBs). After three days, the aggregates that formed were transferred to bacterial petri dishes and exposed to the appropriate concentration of the test chemical for another two days. On day 5, the EBs were placed individually into six wells of a Falcon tissue culture plate. On day 10, the EBs were collected for quantitative polymerase chain reaction (qPCR) detection to observe the expression of related genes.

**qPCR.** The cell samples for analysis were collected on day 10 of the differentiation assay, and total RNA was extracted using an EZNA™ Total RNA kit II (Omega Bio-Tek, Norcross, GA, USA) according to the instructions of the manufacturer. cDNA was synthesized using 1 µg RNA and PrimeScript™ RT Master mix (Takara, Otsu, Japan) as per the instructions of the manufacturer. The reaction mixture was incubated at 37°C for 30 min, followed by 5 sec at 85°C. To verify the undifferentiated marker genes Sox2 and Oct4 and the myocardial-specific marker β-myosin heavy chain (β-MHC), qPCR was performed using SsoAdvanced™ SYBR-Green (Bio-Rad Laboratories, Hercules, CA, USA). qPCR reactions were conducted in a 20-µl mixture that included 10 µl 2X qPCR Master mix, 8 µl nuclease-free water with mouse-specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5'-GCCTTC TCCATGGTGGTGAA-3' and 5'-GCACAGTCAAGGCCGA GAAT-3') and β-MHC (5'-TCCGCAACCGAGAGAATCAG-3' and 5'-TGTCGCCAGAAATTGTGCCTT-3'), and 2 µl cDNA template. The thermal cycle profile consisted of an initial 30-min step at 95°C, followed by 40 cycles of 95°C for 5 sec, and 60°C for 20 min. The CFX Connect Real-Time system and CFX Manager Software (version 2.0; Bio-Rad) were used to collect the PCR data. The RNA levels of each gene were normalized to GAPDH.

**Classification of the embryotoxic potential of the four extracts.** Two permanent mouse cell lines of ESCs and 3T3 cells were used to predict the cytotoxicity of the test compounds using the EST. The concentrations of the compounds that inhibited the proliferation of 50% of the ESCs and 50% of the 3T3 cells (IC<sub>50</sub>ES and IC<sub>50</sub>3T3) were determined with CCK8 assay. In addition, the concentration that inhibited the differentiation of 50% of the ESCs (ID<sub>50</sub>ES) was obtained from the results of qPCR in a differentiation assay. The three endpoints obtained in each experiment were used to calculate linear discriminant functions (I, II, III) for each extract.

I.  $5.9157 \lg(\text{IC}_{50}3\text{T3}) + 3.500 \lg(\text{IC}_{50}\text{ES}) - 5.307 (\text{IC}_{50}3\text{T3-ID}_{50}\text{ES}) / \text{IC}_{50}3\text{T3}-15.72;$

II.  $3.6511 \lg(\text{IC}_{50}3\text{T3}) + 2.3941 \lg(\text{IC}_{50}\text{ES}) - 2.033 (\text{IC}_{50}3\text{T3-ID}_{50}\text{ES}) / \text{IC}_{50}3\text{T3}-6.85;$

III.  $-0.125 \lg(\text{IC}_{50}3\text{T3}) + 1.917 \lg(\text{IC}_{50}\text{ES}) + 1.500 (\text{IC}_{50}3\text{T3-ID}_{50}\text{ES}) / \text{IC}_{50}3\text{T3}-2.67.$

Depending on the variables of the three functions I, II and III, the embryotoxicity of a test compound can be classed as

non-embryotoxic (Class 1), weakly embryotoxic (Class 2) or strongly embryotoxic (Class 3).

The classification criteria were as follows: Class 1: if I>II and I>III; Class 2: if II>I and II>III; and Class 3: if III>I and III>II.

We first used a modified EST to determine the effect of 5-FU, DPH and SAC on differentiation and to classify the embryotoxic potential of each compound, which was revealed to be strong, weak and non-existent, respectively, to verify the predictive validity of the EST we established. Subsequently, the embryotoxic classification of the four herbal extracts was determined with the modified EST.

**Statistical analysis.** The statistical analysis was performed using GraphPad Prism 5 (GraphPad Prism Software, Inc., San Diego, CA, USA). Each data point represented three independent experiments. Data are given as the mean ± SEM. A one-way ANOVA was used to assess the statistical significance. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Verification of the undifferentiated ESCs.** We verified the undifferentiated cells through morphology, AKP staining and qPCR to determine the expression levels of undifferentiated markers in undifferentiated cells. Observed under the microscope, the ESCs were nest-like, compact clones, with clear boundaries (Fig. 1A). They were AKP-positive and deep brown in color (Fig. 1B). The expression levels of the undifferentiated markers Sox2, Oct4 and Nanog were much higher than in the negative control, MEF (Fig. 1C). These results indicated that the ESCs were undifferentiated, and they were used in subsequent experiments.

**Validity check of EST.** The modified EST model was verified by determining the embryotoxicity of 5-FU (Fig. 2), DPH (Fig. 3) and SAC (Fig. 4). Three endpoint values were obtained for each compound. Detailed data on the endpoint values are shown in Table I. We confirmed that 5-FU had strong embryotoxicity, DPH was weakly embryotoxic and SAC was non-embryotoxic.

**Embryotoxicity of four TCM extracts.** The four TCMs (*Rhizoma Atractylodes macrocephala*, *Radix Isatidis*, *Coptis chinensis* and *Flos Genkwa*) were decocted with water, and the EST was performed to determine the embryotoxic potential of the extracts. Three endpoints were obtained for each extract from three independent experiments. The cytotoxicity of the extracts on the OG<sub>2</sub> cells and the 3T3 cells (IC<sub>50</sub>3T3 and IC<sub>50</sub>ES) was determined with a CCK8 assay, and the inhibition of cardiomyocyte differentiation of the ESCs (ID<sub>50</sub>ES; based on the expression levels of the β-MHC gene) was determined by qPCR. The three parameters of each extract were substituted into three linear discriminant functions (I, II and III). Detailed data on the classification of the embryotoxic potential of each extract using the criteria for embryotoxicity are provided in Table I.

***Rhizoma Atractylodes macrocephala.*** In the cytotoxicity assay, the IC<sub>50</sub>3T3 value was 4.82 mg/ml, which was ~1/10

Table I. Three endpoint values of test compounds in the embryonic stem cell test.

Test	IC <sub>50</sub> 3T3	IC <sub>50</sub> ES	ID <sub>50</sub> ES	I	II	III	Criteria	Classification
SAC	2790.00	3009.00	2156.00	15.63	13.60	-9.43	I>II and I>III	1
DPH	89.76	67.87	14.13	-2.23	2.95	-5.16	II>I and II>III	2
5-FU	0.25	0.05	0.07	-27.79	-13.72	1.05	III>I and III> II	3
<i>Atractylodes</i>	4.82	42.42	37.67	30.19	13.40	-16.10	I>II and I>III	1
<i>R. Isatidis</i>	1.76	27.68	15.00	30.00	12.79	-16.74	I>II and I>III	1
<i>C. chinensis</i>	0.40	2.73	1.06	-7.73	-3.89	-5.95	II>I and II>III	2
<i>Flos Genkwa</i>	0.51	0.75	1.15	-11.18	-5.65	-4.77	III>I and III> II	3

Sac, saccharine; DPH, phenytoinum natricum; 5-FU, 5-fluorouracil; *Atractylodes*, Rhizoma *Atractylodes macrocephala*; *R. Isatidis*, Radix *Isatidis*; *C. chinensis*, *Coptis chinensis*.

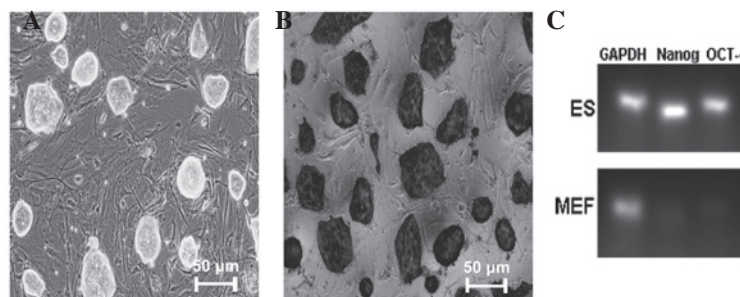


Figure 1. Detection of undifferentiated state of embryonic stem cells (ESCs). (A) Mouse embryonic stem cell growth in the feeder layer (magnification, x100); (B) Alkaline phosphatase staining of ESCs (magnification, x100); (C) Expression of undifferentiated Oct4 and Nanog genes of ESCs. MEF, mouse embryonic fibroblast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

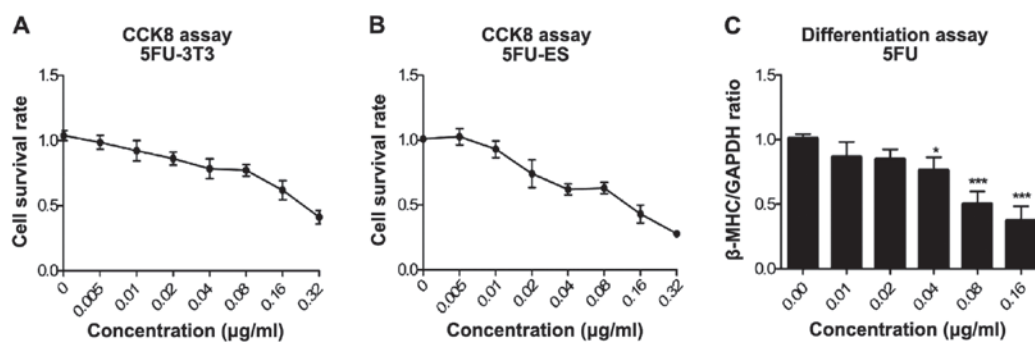


Figure 2. Embryonic stem cell (ESC) test for 5-fluorouracil (5-FU). Cytotoxicity assay with 3T3 cells (A) and ESCs (B). Differentiation ESC assay (C). Relative levels of  $\beta$ -myosin heavy chain expression and cytotoxicity are represented as a percentage of the solvent control (control=100%). Values are means  $\pm$  SEM (n=3). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

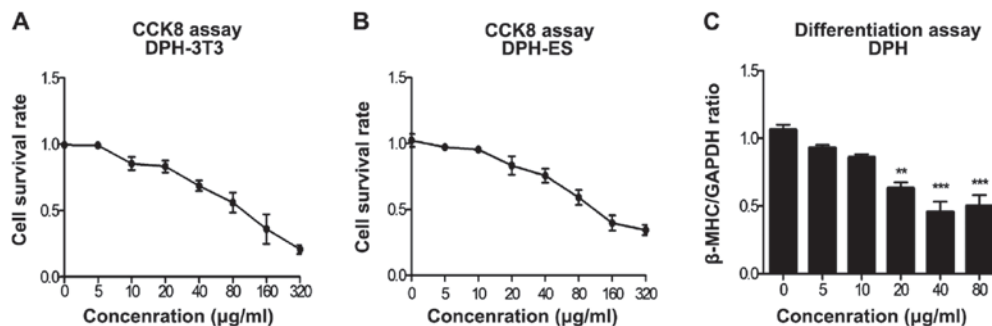


Figure 3. Embryonic stem cell (ESC) test for phenytoinum natricum (DPH). Cytotoxicity assay with 3T3 cells (A) and ESCs (B). Differentiation ESC assay (C). Relative levels of  $\beta$ -myosin heavy chain expression and cytotoxicity are represented as a percentage of the solvent control (control=100%). Values are means  $\pm$  SEM (n=3). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.



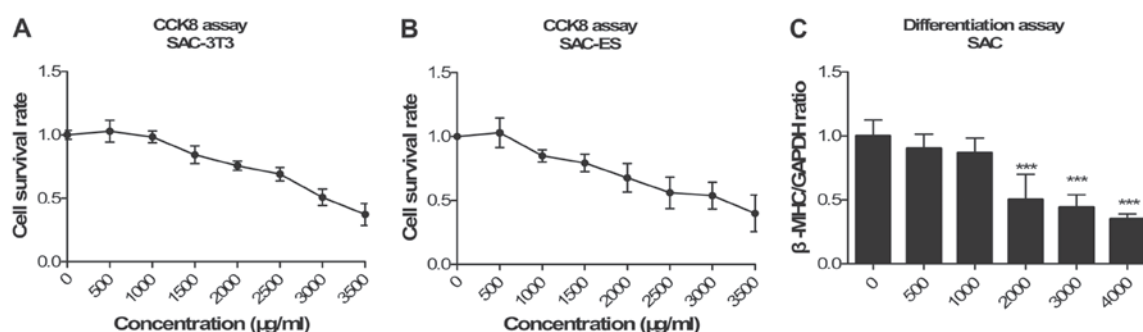


Figure 4. Embryonic stem cell (ESC) test for saccharin (SAC). Cytotoxicity assay with 3T3 cells (A) and ESCs (B). Differentiation ESC assay (C). Relative levels of  $\beta$ -myosin heavy chain expression and cytotoxicity are represented as a percentage of the solvent control (control=100%). Values are means  $\pm$  SEM (n=3). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

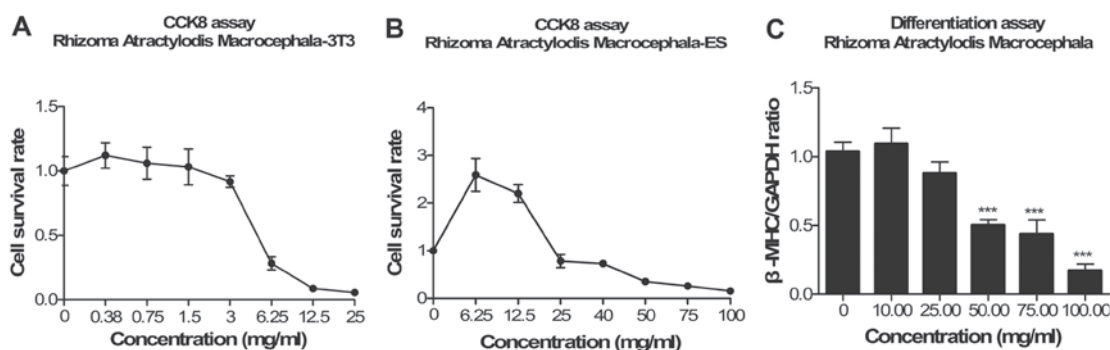


Figure 5. Embryonic stem cell (ESC) test for *Rhizoma Atractylodis macrocephala*. Cytotoxicity assay with 3T3 cells (A) and ESCs (B). Differentiation ESC assay (C). Relative levels of  $\beta$ -myosin heavy chain expression and cytotoxicity are represented as a percentage of the solvent control (control=100%). Values are means  $\pm$  SEM (n=3). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

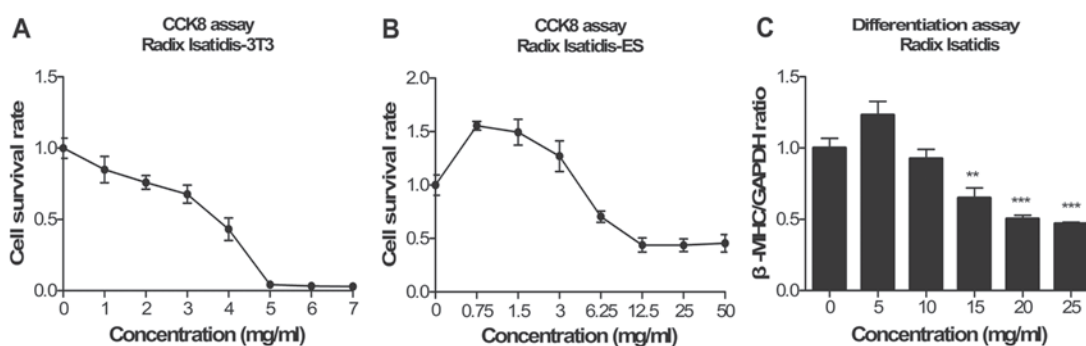


Figure 6. Embryonic stem cell (ESC) test for *Radix Isatidis*. Cytotoxicity assay with 3T3 cells (A) and ESCs (B). Differentiation ESC assay (C). Relative levels of  $\beta$ -myosin heavy chain expression and cytotoxicity are represented as a percentage of the solvent control (control=100%). Values are means  $\pm$  SEM (n=3). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

that of the  $IC_{50}$ ES value (42.42 mg/ml; Fig. 5A and B). The  $ID_{50}$  value for *Rhizoma Atractylodes macrocephala* was 37.67 mg/ml (Fig. 5C). It promoted the survival of ESCs and led to cardiac differentiation of ESCs at a low concentration. Thus, it was defined as a non-embryotoxic compound.

*Radix Isatidis*. ESCs increased at a low *Radix Isatidis* concentration. As the concentration increased, the survival rate of the ESCs declined. The proliferation of the 3T3 cells was suppressed at a low concentration, whereas the proliferation of the ESCs was promoted (Fig. 6A and B). The  $IC_{50}$  values were 1.76 and 27.68 mg/ml for the 3T3 fibroblasts

and the ESCs, respectively. The expression levels of  $\beta$ -MHC decreased in a concentration-dependent manner following exposure to *Radix Isatidis* extract (Fig. 6C). The  $ID_{50}$  values were 15 mg/ml. *Radix Isatidis* extract was classified as non-embryotoxic.

*Coptis chinensis*. In both cell lines, the *Coptis chinensis* extract inhibited the survival of cells in a dose-dependent manner (Fig. 7A and B). The cytotoxic sensitivity of the 3T3 fibroblasts was much higher than that of the ESCs exposed to *Coptis chinensis* extract. The  $IC_{50}$  values were 0.398 and 2.73 mg/ml for the 3T3 fibroblasts and the ESCs, respectively.

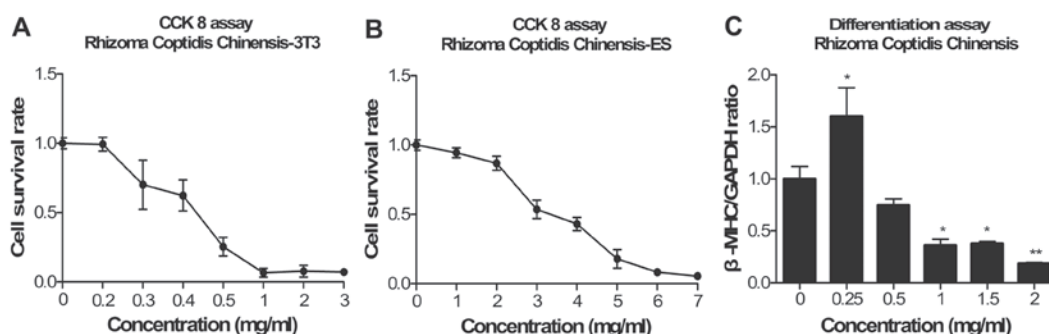


Figure 7. Embryonic stem cell (ESC) test for *Coptis chinensis*. Cytotoxicity assay with 3T3 cells (A) and ESCs (B). Differentiation ESC assay (C). Relative levels of  $\beta$ -myosin heavy chain expression and cytotoxicity are represented as a percentage of the solvent control (control=100%). Values are means  $\pm$  SEM (n=3). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

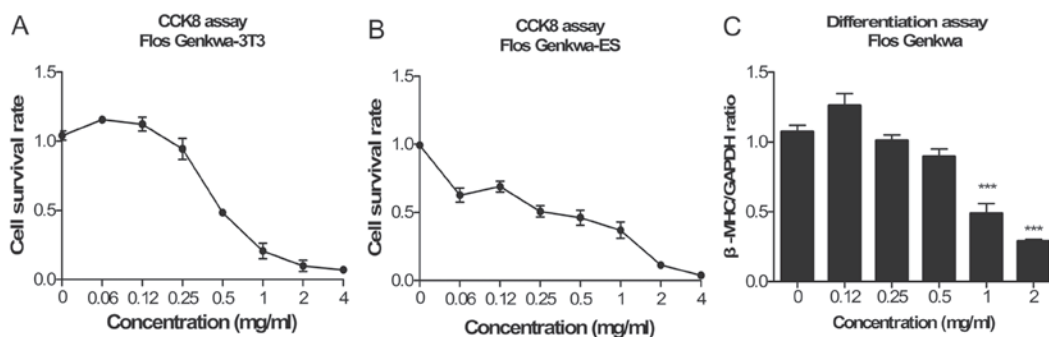


Figure 8. Embryonic stem cell (ESC) test for *Flos Genkwa*. Cytotoxicity assay with 3T3 cells (A) and ESCs (B). Differentiation ESC assay (C). Relative levels of  $\beta$ -myosin heavy chain expression and cytotoxicity are represented as a percentage of the solvent control (control=100%). Values are means  $\pm$  SEM (n=3). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

The ID<sub>50</sub> value was 1.06 mg/ml. The *Coptis chinensis* extract was defined as weakly embryotoxic.

**Flos Genkwa.** The 3T3 cells and ESCs were both sensitive to *Flos Genkwa*. The IC<sub>50</sub> values for the 3T3 cells (0.51 mg/ml) and those for the ESCs (0.75 mg/ml) were below 1 mg/ml (Fig. 7A and B). The ESCs were slightly more sensitive than the 3T3 cells when the concentration was under 0.25 mg/ml. However, as the concentration was increased, the opposite trend was observed. The value of ID<sub>50</sub> was 1.15 mg/ml. *Flos Genkwa* was classified as strongly embryotoxic.

## Discussion

The EST is an *in vitro* tool to assess the developmental toxic potency of test compounds in early development. Its coincidence rate is 78% compared with *in vivo* test results (21). The EST has been successfully used to classify the embryotoxicity of a large number of chemical compounds (25). It is now starting to be used to estimate the embryonic toxicity of natural compounds of Chinese medicinal herbs. Using the EST, bisphenol A and genistein were classified as weakly embryotoxic and strongly embryotoxic, respectively (23). Baicalin, an active constituent of *Radix Scutellariae*, demonstrated weak embryotoxicity based on the EST (24).

In the current study, we attempted to apply the EST to aqueous extracts of Chinese herbs. A mouse embryonic stem cell line, OG<sub>2</sub>, was used as a substitute for the D3 cell line in

the EST, and the embryotoxicity of 5-FU, DPH and SAC was evaluated to verify the reliability of our findings. Our results revealed that 5-FU, DPH and SAC were strongly embryotoxic, weakly embryotoxic and non-embryotoxic, respectively, which is in accordance with the results of the EST validation test. This indicated that the findings were reliable when the D<sub>3</sub> cell line was replaced with the OG<sub>2</sub> cell line in the EST. We then used the EST to detect the embryotoxicity of the four selected tested Chinese herbal medicine aqueous extracts.

*Rhizoma Atractylodes macrocephala* is commonly used in TCM for pregnant women to treat abnormal fetal movement. In safety evaluation research, water-soluble extract of *Rhizoma Atractylodes macrocephala* revealed no genotoxicity based on four genotoxicity tests (26). Our *in vitro* results revealed that it exhibited no embryotoxicity.

*Radix Isatidis* is a valuable Chinese medicine with antibacterial and antiviral activities as well as an excellent safety profile (27). However, it was demonstrated that water boiled juice of *Isatic tinctoria* L. caused micronuclei and sperm abnormalities in mice, and it was deemed a potential mutagen (28). Our results indicated that *Radix Isatidis* has no embryotoxicity.

A survey of Chinese herbal medicines used during pregnancy previously revealed that *Coptis chinensis* was one of the five most commonly used Chinese herbal medicines (4). It exhibited acute toxicity at LD<sub>50</sub> under 10 g/kg in mice (29). Berberine, one of the major constituents of *Coptis chinensis*, was also reported to exhibit genotoxicity (30). The present

study suggests that *Coptis chinensis* has weak embryotoxic potential.

Since China's one-child family planning policy was enacted, *Flos Genkwa* has been widely used in abortion. No studies have investigated the embryonic toxicity of *Flos Genkwa*, although it is considered a toxic Chinese medicine. According to our results, *Flos Genkwa* was strongly embryotoxic.

In conclusion, the results of the present study suggest that extracts of *Rhizoma Atractylodes macrocephala* and *Radix Isatidis* are non-embryotoxic, whereas those of *Coptis chinensis* and *Flos Genkwa* are weakly embryotoxic and strongly embryotoxic, respectively. Our study potentially offers valuable information that may be used to expand the application of EST to the field of TCM. Moreover, as ESCs are pluripotent, herbal remedies with an ability to induce cell differentiation may be discovered. Such a finding would be of great significance in the field of TCM.

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