



**Good Practice in Traditional Chinese Medicine Research in  
the Post-genomic Era**

**GP-TCM**

**223154**

**D5.10**

**Recommendations on best practice in CHM in animal models  
of disease**



<b>Document description</b>	
Name of document	Recommendations on best practice in CHM in animal models of disease
Abstract	The document is focused on giving examples which have been found in scientific journals
Document identifier	D5.10
Document class	Deliverable
Version	Final
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Date of creation	20 <sup>th</sup> March 2011
Date of last modification	30 <sup>th</sup> June 2011
Status	Final
Destination	European Commission
WP number	WP5



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## 1 INTRODUCTION

This document has been developed for answering the central question “What good practice should be followed in animal studies of Chinese herbal Medicine (CHM)?”.

In the WP5 kick-off meeting it was decided to start with a preliminary review focused on the current status of CHM studies in animals. In a second step, it was decided that an in-depth review in cancer should be undertaken and would be considered as a significant sample of the state-of-the-art in CHM studies in animals: according to MedLine, oncology is one of the most active medical areas in CHM in the last 10 years (see Report on Deliverable D5.4, Volume I). After reviewing the scientific literature on CHM in animal models of cancer, we have found that the experimental work reported in 59% of the papers published in English was below the minimal criteria of quality (see Report on Deliverable D5.4, Volume II). The current D5.10 report includes recommendations to improve the quality of research from the point of view of experimental design.

The analysis reported above was focused on the animal model itself and the experimental design, without taking into account the specific problems of CHM preparation. As stated in the Handbook on Good Practice in the reporting of CHM experimental work (WP4 deliverable), crude herbal drugs and particularly Chinese formulae are natural products and their chemical composition varies depending on several factors, such as the geographic source of the plant material, the climate in which the plant was grown, and time of harvest. Commercially available herbal medicinal products also vary in their composition, both quantitatively and qualitatively, from batch to batch. Even when herbal products are standardized for content of known active or marker compounds there is variation in the concentrations of other constituents that can result in differences in pharmacological activity. Also, preparation methods vary significantly (including the means of extraction, if any; whether the material is heated for a prolonged period, etc) which could also contribute to variability in chemical composition and hence pharmacological activity.

We have reviewed the botanical origin, processing and extraction procedures in the papers on CHM studies in animals and we have found that in most cases the descriptions are insufficient to ensure the quality and reproducibility of the studies. Accordingly, our report on best practice in CHM in animal models of disease also includes recommendations to assure a consistent and acceptable quality herbal product.

## 2 METHODS

We have analyzed research into CHM documented in MedLine in the field of animal studies of cancer. We have chosen those references in English which involved ‘true’ CHM preparations (i.e. herbal mixtures of 3 or more herbs prepared following the principles of CHM) and which were published within the last 10 years. We retrieved 31 scientific articles, which represent more than 90% of the references in English found in MedLine. They were first analyzed (see Table I in the Results section) for botanical origin, processing and extraction procedures as well as for the description of the animal model and the use of conventional medicine for comparison of efficacy.

Taking into account the results of this analysis and the recommendations of the GP-TCM Handbook on Good Practice in the Reporting of CHM Experimental Work, the IUPAC and important reviews on the quality of experimental research in animals, we propose a list of recommendations on best practice in CHM in animal models of disease which are based upon definite examples found in the reviewed literature.

### 3. RESULTS

Table I shows the results on the analysis of 31 scientific articles, which represent more than 90% of the references in English found in MedLine in the last 10 years on 'true' CHM in animal models of cancer. *The list of 31 articles is in References section. Due to this, the other references in this deliverable are indicated as footnotes*

**Table I: Methodological aspects of scientific articles on CHM in animal models of cancer published in English in the last 10 years**

	STATED/DONE		NO STATED/NOT DONE		TOTAL	
	TOTAL	%	TOTAL	%	TOTAL*	%
<b>1-BOTANICAL SOURCE*</b>						
HARVESTED/ COMMERCIAL PREPARATION	9	40,91	13	59,09	22	100,00
AUTHENTICATION	4	18,18	18	81,82	22	100,00
KEEP RECORDS?	0	0,00	22	100,00	22	100,00
PHYSICAL PARAMETER**	0	0,00	22	100,00	22	100,00
CHROMATOGRAPHIC/SPECTROSCOPIC	1	4,55	21	95,45	22	100,00
MICROBIOLOGICAL CONTAMINATION	0	0,00	22	100,00	22	100,00
PESTICIDE RESIDUE ANALYSIS	0	0,00	22	100,00	22	100,00
HEAVY METAL ANALYSIS	0	0,00	22	100,00	22	100,00
<b>2-PROCESS*</b>						
CLEANING	0	0,00	22	100,00	22	100,00
CUTTING FRESH/DRY***	0	0,00	22	100,00	22	100,00
ROASTING/ BROILING	0	0,00	22	100,00	22	100,00
<b>3-EXTRACTION*</b>						
PROCEDURE	10	45,45	12	54,55	22	100,00
CHARACTERIZATION***	3	13,64	19	86,36	22	100,00
<b>4-CONTROL</b>						
CONVENTIONAL MEDICINE	8	25,81	23	74,19	31	100,00
<b>5-MODEL</b>						
SPECIES	31	100,00	0	0,00	31	100,00
SEX	22	70,97	9	29,03	31	100,00
AGE	21	67,74	10	32,26	31	100,00
WEIGHT	21	67,74	10	32,26	31	100,00
RANDOM SELECTION	13	41,94	18	58,06	31	100,00
	1	3,23	30	96,77	31	100,00
EUTHANASIA PROCESS DESCRIBED	7	22,58	24	77,42	31	100,00
ETHICAL APPROVAL GRANTED	7	22,58	24	77,42	31	100,00

\* 22 out of the 31 articles analyzed did not use commercially available proprietary products. Items 1, 2 and 3 are specific for these 22 articles.

\*\*Physical tests<sup>1</sup> include organoleptic evaluation (sensory characters such as taste, appearance, odor, feel of the drug, etc.), viscosity, moisture content, pH, disintegration time, friability, hardness, flowability, sedimentation, and ash value.

\*\*\*Unless cutted in fresh or dry form, crude traditional Chinese drugs should be moistened to soften the drug prior to cutting.

<sup>1</sup> Anon. *General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine*, p. 4, World Health Organization, Geneva (2000).



22 out of the 31 articles analyzed (71%) did not use commercially available CHM preparations and therefore they were analyzed for botanical origin, processing and extraction procedures. All the 31 articles were analyzed for the use of conventional medicine for comparison of efficacy and for the description of the animal model.

Results in Table I indicate that important methodological aspects are not stated or done in the analyzed papers. These aspects are relevant for standardization and reproducibility of the experiments, for an adequate experimental design and for ethical issues. In consequence, the following recommendations and examples on best practice are proposed.

#### **4. RECOMMENDATIONS ON BEST PRACTICE (Summary)**

These recommendations are meant to promote best practice in the methodological issues indicated in Table I and they are based upon the IUPAC protocols and the GP-TCM Handbook on Good Practice in the Reporting of CHM Experimental Work for assuring a consistent and acceptable quality of herbal product, and in the review published by Kilkenny C et al (see footnote 4) for experimental design-related aspects.

*To assure a consistent and acceptable quality herbal product*

General records

stage of collection, parts of the plant collected, regional status, details of any voucher specimen

Authentication of the herbal raw material

taxonomic, macroscopic & microscopic

Standardization (to provide quantitative and semiquantitative information about the main active constituents or marker compounds present in the crude drug or herbal products)

Chromatographic and sophisticated modern techniques: spectroscopic evaluation UV–vis spectrophotometry, TLC, HPTLC, HPLC-mass spectrometry, NMR, etc

Physical parameters: organoleptic evaluation, viscosity, moisture content, pH, disintegration time, friability, hardness, flowability, sedimentation and ash value.

Microbiological contamination

Pesticide residue

Heavy metal analysis

Extraction

Solvent used and ratio, time, temperature, yield

When reporting the results of a study, it is necessary to indicate all the details related with the aspects described above. In addition, in the case of processed plant and/or mixtures of plants or of a proprietary product, the batch number of the herbal product should be clearly indicated.

*To assure an adequate report of the animal studies themselves*

It is necessary to indicate

Animal strain, sex, sample size

Randomisation and blinding

Euthanasia procedures

Ethical approval

It is recommended to include in the experimental design

Comparison with a conventional medicine

Long term studies

## 5. RECOMMENDATIONS ON BEST PRACTICE (Full version)

### 5.1 Recommendations based upon the IUPAC protocols<sup>2</sup>

In order to assure a consistent and acceptable quality herbal product, care should be taken right from the identification and authentication of herbal raw materials to the verification process of final product. The following parameters are recommended.

**1. Authentication.** The first stage is identification of the plant species or botanical verification by the currently accepted Latin binomial name and synonyms. The steps involved in authentication are taxonomic, and macroscopic and microscopic studies. Records should be maintained for stage of collection, parts of the plant collected, regional status, botanical identity such as phytomorphology, microscopical, and histological analysis, taxonomical identity, etc.

*Poncirus trifoliata* (L.)Raf, *Akebia Trifoliata* Koidz, *Citrus medica* var. *sarcodactylis* Swingle and *Saussurea lappa* were purchased from Shanghai Pharmaceutical Co., Shanghai, China, and were authenticated by Prof. Bo-Wen Qian, Department of Traditional Chinese Medicine, Shanghai University of Chinese Traditional Medicine, Shanghai, China [6].

The herbs used in the current study were supplied in a dry form by the China Medical College Hospital, Taichung, Taiwan. Their identification was authenticated by experts in pharmacognosy [28]

It was prepared as a lyophilized-dry powder of hot water extracts from 17 species of medical herbs consisting of *Coptis chinensis* FRANCH (5.6 g), *Cimicifuga heracleifolia* KOMAR (8.6 g), *Scutellaria baicalensis* GEORGI (22.9 g), *Gentiana scabra* BUNGE. (14.3 g), *Trichosanthes cucumeroides* (SER.) MAXIM. (14.3 g), *Phellodendron amurense* RUPR. (22.9 g), *Anemarrhena asphodeloides* BUNGE. (14.3 g), *Platycodon grandiflorus* (JACQ.) (14.3 g), *Laminaria japonica* ARESCH. (14.3 g), *Bupleurum chinese* DC. (14.3 g), *Glycyrrhiza uralensis* FISCH. (8.6 g), *Sparganium stoloniferum* BUCCH. (8.6 g), *Curcuma aeruginosa* ROXB (8.6 g), *Forsythia suspense* (THUMB.) VAHL (8.6 g), *Pueraria lobata* OHWI (8.6 g), *Paeonia lactiflora* PALL. (5.6 g) and *Angelica sinensis* (OLIV.) DIELS. (5.6 g). They were purchased from a local herb store. The authenticity of the plant species was confirmed by Doctor MH Yen of Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan [4].

A quantity of TJ-10 preparation weighing 7.5 g contained 4.0 g of extracts of 5.0 g of *Bupleuri Radix*, 4.0 g of *Pinelliae Tuber*, 2.0 g of *Scutellariae Radix*, 2.0 g of *Glycyrrhizae Radix*, 2.0 g of *Cinnamomi Cortex*, 2.0 g of *Paeoniae Radix*, 2.0 g of *Zizyphi Fructus*, 2.0 g of *Ginseng Radix*, and 1.0 g of *Zingiberis Rhizoma* [22].

KQR, composed of *Radix morinda officinalis* 15 g, *eupolyphaga seu steleophaga* 15 g, *pheretima* 15 g, *Radix et Rhizoma Rhei* 5 g, *Ramulus Cinnamomi* 5 g, and *caulis trachelospermi* 20 g, was provided by the Fujian Tongchun Medicament Stock Company, China [5].

Jinlongshe (JLS) granules are an oral Chinese medicine compound, consisting of Chinese herbs (*Rhizome Arisaema*, *Rhizoma Pinelliae*, *corium stomachium galli*, *Radix Glycyrrhizae preparata*, etc.), developed by our department based on TCM theories of “phlegm” as the key factor in preventing and treating gastric cancer. Crude JLS granules were purchased from Leiyunshang Company, Shanghai, China. An aqueous extract of JLS granules at a concentration of 6 g/mL of raw materials was provided by Shanghai Changzheng Hospital [29].

Ingredients of ACNO(Latin)(patent a135325): *Panax ginseng* (9.5%), *Poria cocos* (5.7%), *Atractylodes macrocephala*(5.7%), *Anglica sinensis* (5.7%), *A. membranaceus* (5.7%), *Curcuma zedoaria*(4.7%), *Scutellaria baicalensis* (5.7%), *Phellodenron chinense*(4.7%), *Coptis chinensis*(5.7%), *Glycyrrhiza uralensis*(5.7%), *Crataegus pinnatifida* (4.7%), *Hordeum vulgare*(1.9%), *Salvia miltiorrhiza*(4.7%), *Schisandra chinensis* (5.7%), *Hedyotis. diffusa* (6.6%)., *Ophiopogon japonicas* (4.7%), *Lobelia chinensis lour* (4.7%), *Scutellaria barbaba* (5.7%), *Massa fermentata medicinalis* (1.9%). *P. ginseng* was bought from Hui Nan Ginseng (Ji Lin province, China). *S. barbaba* was purchased from KPC

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<sup>2</sup> Mosihuzzaman M and Choudhary MI Protocols on safety, efficacy, standardization and documentation of herbal medicine *Pure Appl. Chem.* 80, 2195–2230, 2008



Products (Taiwan, China). All other compounds were bought from Mayway (Oakland, CA) ACNO was dissolved in boiling water [3].

Bing De Ling solution consists of Astragalus root (*Astragalus membranaceus*), rhubarb root (*Rheum palmatum*), white atractylodes (*Atractylodes macrocephala*), isatis root (*Isatis tinctoria*), scutilliar root (*Scutellaria baicalensis*), dogberry (*Cornus officinalis*), and shield fern root (*Dryopteris erassirhizoma*) at a concentration of 0.121 g/ml of water [26].

Huqi San was made of eight medicinal herbs containing glycopival granules by gently boiling the herbal drugs, such as Ramulus Visci, Radix Astragali seu Hedysari, Radix Curcumae and Radix Salviae Miltiorrhizae in distilled water for 60 min to reduce the volume [12].

Chinese medicine *Gekko* was purchased from Anhui Bozhou Yonggang Co. Ltd. They were identified *Gekko japonicus*. The whole dry *Gekko* were ground to fine powder and diluted in suspension using 0.2% carboxymethyl cellulose (CMC) [15] (*Gekko* *gekko* is an animal used as a valued traditional Chinese medicine; in this case the CHM includes non-botanical components).

Zyflamend was provided by the manufacturer (NewChapter, Brattleboro, VT) in a defined olive oil-based suspension of 10 concentrated anti-inflammatory herbs. For all in vitro experiments, the product was mixed with dimethyl sulfoxide at a 1:1 dilution and then further diluted (1:1000) in tissue culture medium. Concentrations of this liquid herbal product are described as microliter of Zyflamend per milliliter of tissue culture media [27].

**2. Physical parameters** Physical tests include organoleptic evaluation (sensory characters such as taste, appearance, odor, feel of the drug, etc.), viscosity, moisture content, pH, disintegration time, friability, hardness, flowability, sedimentation, and ash value.

*ZYD88 preparation and chemicals.* ZYD88 is composed of 6 Chinese medicinal herbs (Table I), and is processed by a proprietary technology that was developed by the Changsha Cihang Research Institute of Materia Medica (Changsha, China). Briefly, the entire processing of ZYD88 including such parameters as temperature, humidity, pH, viscosity, pressure, extraction time, and yield, was consistently monitored by an automatic feedback system that consists of a central control station with several peripheral sensors, regulators and quality control detectors [2].

**3. Chromatographic and sophisticated modern techniques of standardization** such as spectroscopic evaluation UV–vis spectrophotometry, TLC, HPTLC, HPLC, NMR, near infrared spectroscopy provide quantitative and semiquantitative information about the main active constituents or marker compounds present in the crude drug or herbal products. Markers play an important role in fingerprinting of herbs. Quality of drug can also be assessed by chromatographic fingerprint.

To ensure consistency of MINA-05 components between batches, HPLC profiling and liquid chromatography–mass spectrometry (LC-MS) analysis based on markers selected from the Chinese Pharmacopeia were performed [23].

The presence of anthraquinone ingredients (emodin and rhein) and tetrahydropalmatine in each preparation was analyzed by thin-layer chromatography, using purified emodin, rhein and tetrahydropalmatine as the standards, respectively. The concentration of emodin in each preparation was determined by an absorbance at 510 nm, and the average amount of emodin in 5 lots of ZYD88 analyzed was  $0.622 \pm 0.056$  [standard deviation (SD)]% (9). A liquid extract of ZYD88 at a concentration of 1 g of total raw herbs per ml was prepared and used for the experiments. The concentration was expressed as the weight of dry raw herbs in volume (w/v) [2].

For in vivo experiments, this formulation was dissolved in distilled water and then diluted to the appropriate doses before oral administration. HPLC pattern analysis, the so-called ‘**fingerprint**’ method, was performed to assess the homogeneity of the formulation and to prepare batches of constant formulation and efficacy, as described previously. Fig. 1 shows the HPLC profiles of Bojung-bangamtang in terms of single monitor (220 nm), contour plot (190–420 nm) and three-dimensional pattern using a photodiode array system as detector. Five peaks, c, d, e, f and h, were identified by comparison with the retention time and the UV spectra of standard compounds [10].

The composition of WCA includes *Atractylodes macrocephala* koidz., *Poria cocos* (Schw.) Wolf, *Glycyrrhiza uralensis* Fisch., Rehd. Et Wils., and *Prunella vulgaris* L. The preparation of WCA decoction has been described previously, and the concentration of the decoction was 120 g/L. High Performance Liquid Chromatography (HPLC) was used for monitoring the stability of the decoction. Lichrospher-C18 column at 25°C was used. The mobile phase consisted of methyl alcohol/water with a linear gradient as follows: methyl alcohol, 5, 5, 70, 100, 100%; water, 95, 95, 30, 0, 0%; at 0, 5, 15, 35 and 40 min, respectively, and the flow rate was 1 mL/min. The detection was performed at UV 280 nm (Figure 1) [31].

**4. Microbiological** Microbiological contamination can be measured according to parameters methods described in the *Romanian Pharmacopoeia*, as well as in the *British Pharmacopoeia*. Microbiological analysis includes analysis of limits of *E. coli* and molds, total viable aerobic count, total enterobacteria and their count, aflatoxin analysis.

**5. Pesticide residue** Standard limits of pesticides have been set by WHO and analysis FAO (Food and Agricultural Organization). Some common pesticides that cause harm to human beings, such as DDT, BHC, toxaphene, and aldrin, should be analyzed.

**6. Heavy metal analysis** Toxic metals such as Cu, Zn, Mn, Fe, and particularly Cd, As, Pb and Hg should be analyzed. In the analysis of metals, their speciation is to be taken into consideration.

No examples have been found on these 3 recommendations in any of the scientific articles analyzed.

## 5.2. Recommendations of the GP-TCM Handbook on Good Practice in the Reporting of CHM Experimental Work

These recommendations are more exigent than the IUPAC ones. We have found scarce examples which meet at least in part the level of quality required. They are indicated below, after the recommendations.

### Methods – (plant description)

There are GENERAL criteria which apply to all articles and others which refer to the specific type of preparation used.

General: The methods should describe:

- The method of authentication of the herbal raw material indicated;

*Examples in section 4.1*

- Solvent used and ratio for the extraction;

- Time of extraction;

... decocted three times with boiling distilled water for 1 h [6].

Each recipe (200 g) was decocted three times with 1 l boiling distilled water for 2 h [4].

After being soaked for 1-2 h and boiled for 30 min, the decoction was filtered. Once again, water of about 3-5 times as the herbs was added, decocted and boiled for 20 min before the second filtration [1].

Fifty grams of the ground material were added to 1000 ml of distilled water under reduced pressure for 6 hours at room temperature. The insoluble residue was further extracted with boiling water under 1 atm for 3 hours [28].

... were boiled with distilled water (2L<sub>r</sub> 2) at 100 °C for 2 h [10].

- Temperature of extraction;

TJ-10 was stable during pelleting, because TJ-10 powder was extracted with boiling water at 100°C, but TJ-10 was pelleted with Other materials at 80±100°C [22].



After grinding, it was added into 80 °C distilled water to solve [5].

Bojung-bangam-tang is composed of nine crude drugs (Table 1) and was prepared as follows. The nine dried and pulverized medicinal plants (5-day dose) were boiled with distilled water (2L<sub>r</sub> 2) at 100 jC for 2 h [10].

- Yield of extraction;

The yield of liqi was approximately 20% [6].

The average yield obtained for SZKJT was 20.7% [4].

The final yield of XZT powder was 32.4 g [28].

... to produce 186.92 g of powder (Yield 31.15%) [10].

An adequately standardized procedure which recapitulates several recommendations is:

*ZYD88 preparation and chemicals.* ZYD88 is composed of 6 Chinese medicinal herbs (Table I), and is processed by a proprietary technology that was developed by the Changsha Cihang Research Institute of Materia Medica (Changsha, China). Briefly, the entire processing of ZYD88 including such parameters as temperature, humidity, pH, viscosity, pressure, extraction time, and yield, was consistently monitored by an automatic feedback system that consists of a central control station with several peripheral sensors, regulators and quality control detectors [2].

- Details of any voucher specimen must be included;

*We have not found any examples*

- The test material should have been subjected to simple chemical constituent profiling and/or complex fingerprinting. The methods used should be described as well as the service, if applicable;

*Examples in section 4.1*

- The material should have been standardised (by what process and by whom), if appropriate and possible.

*Examples in section 4.1*

- Specific: In the case of unprocessed plant and/or mixtures of plants, there are two extra criteria:

- The herbal product name should be clearly indicated;
- The part of the plant used to make the product should be specified.

- Specific: In the case of processed plant and/or mixtures of plants, there are five extra criteria and guidelines:

- The processed products names or the extracts names and the name of the manufacturer of the products should be indicated;
- The batch number of the herbal products should be indicated;
- The parts of the plants used to make the product or the extract should be specified;

Where applicable:

- The type of preparation to make the test material should be described;
- The yield of the extraction to make the test material should be indicated.

Specific: In the case of a proprietary product, there are five extra criteria:

- The proprietary product name or the extract name and the name of the manufacturer should be indicated;
- The batch number of the product should be indicated;
- The part of the plants used to make the product or the extract should be indicated;

Where applicable:

- The type of preparation to make the test material should be described;
- The yield of the extraction to make the test material should be indicated.

*We have not found any example which meet these recommendations*

We end this part with an example from the Kampo medicine which recapitulates most recommendations indicated above.

Medicinal herbs of Shikunshito-Kamiho (SKTK) were purchased from Koryo Company of Traditional Crude Drugs (Seoul, Korea) and kindly authenticated by Dr. Deok-Kyun Ahn, Professor of Department of Herbology, Oriental Medical College, Kyunghee University. SKTK (40.0 g) consists of eight crude drugs (*Panax ginseng* C. A. MEYER, *Astractylodes macrocephala* KOIDZUMI, *Poria cocos* WOLFF, *Glycyrrhiza uralensis* FISCH, *Prunella vulgaris* var. *lilacina* NAKAI, *Sargassum pallidum* (TURN.) C. AG., *Laminaria japonica* ARESCH and *Ostrea gigas* THUBERG). The composition of SKTK, botanical origins, crude drugs, voucher number, harvesting, place and dose of each herb are shown in Table 1. Voucher specimens were deposited in the herbarium of the Museum of Materia Medica, Oriental Medical College, Kyunghee University, Seoul, Korea. The qualities of medicinal herbs were controlled by Korean Pharmacopeia VII which regulates the botanical origin, the crude drug, loss of drying, total ash, test of foreign matter, acid insoluble ash, extract content, essential oil content and microscopic examination)

**Preparation of SKTK Extract** One and two tenths kilograms of SKTK was mixed and boiled with regular water. The boiled solution was concentrated with rotary evaporator (Model NE-1, Japan) and dried with freeze dryer (Model FD- 1, Japan) to obtain 195 g of SKTK extract. The yield of water extract of SKTK was 16.25% (w/w) in terms of the dried medicinal herbs. Usually, the daily dose of SKTK extract in the treatment of human is 19.5 g three times a day. Thus, the daily dose ratio for a mouse was determined as 0.5% and 1.5% of regular diet considering daily food intake (approximately 4 g) and drug susceptibility. **Identification of Major Chemical Components in SKTK by HPLC Profile Analysis** Chemical components such as glycyrrhizin, ginsenoside Rb1, Rg1 and ursolic acid were identified in SKTK by HPLC analysis under the following conditions. Glycyrrhizin : Extraction solvent, 70% ethanol; column, Nucleosil 120-5 C18 (4.63250 mm), mobile phase, H<sub>2</sub>O:CH<sub>3</sub>CN:tetrabutylammonium bromide (620 ml : 380 ml : 1.5 g); flow rate, 1.5 ml/min; detector, Waters<sup>TM</sup> 486 (254 nm); room temperature, 119180. Ginsenoside Rb1 : Extraction solvent, 70% methanol; column, m-Bondapak C18 (4.63250 mm), mobile phase, H<sub>2</sub>O:CH<sub>3</sub>CN (71 : 29); flow rate, 2.0 ml/min; detector, Waters<sup>TM</sup> 486 (203 nm); room temperature, 139540. Ginsenoside Rg1 : Extraction solvent, 70% methanol; column, m-Bondapak C18 (4.63250 mm), mobile phase, H<sub>2</sub>O:CH<sub>3</sub>CN (81 : 19); flow rate, 2.0 ml/min; detector, Waters<sup>TM</sup> 486 (203 nm); room temperature, 139540. Ursolic acid : Extraction solvent, methanol; column, m-Bondapak C18 (4.63250 mm), mobile phase, H<sub>2</sub>O:CH<sub>3</sub>CN: MeOH (6 : 12 : 5); flow rate, 1.5 ml/min; detector, Waters<sup>TM</sup> 486 (213 nm); room temperature<sup>3</sup>.

### 5.3. Recommendations on best practice in animal studies

These recommendations are meant to avoid important flaws found in the reviewed scientific articles (see Introduction). They are based on the conclusions of review articles on the quality of animal studies<sup>4</sup>

- Information on species (in the case of primates) or strain of animals, sex of the animals, the age of the animals, their weight, the number of animals

Eighty Sprague-Dawley (SD) male rats, aged 6–7 weeks, were fed 4NQO in their drinking water for 36 weeks. Experimental Animal Administrative Committee of Shanghai granted ethical approval for the animal experiments [9].

Sixty 6- to 7-week-old male BALB/c nu/nu (nude) mice [23].

<sup>3</sup> Bong-Ha YOO, Beom-Han LEE, Jin-Sung KIM, Nam-Jae KIM, Sung-Hoon KIM, Ki-Won RYU. Effects of Shikunshito-Kamiho on Fecal Enzymes and Formation of Aberrant Crypt Foci Induced by 1,2-Dimethylhydrazine. Biol. Pharm. Bull. 24(6) 638–642 (2001) Vol. 24, No. 6

<sup>4</sup> Kilkenny C, Parsons N, Kadyszewski E, Festing MFW, Cuthill IC, et al. (2009) Survey of the Quality of Experimental Design, Statistical Analysis and Reporting of Research Using Animals. PLoS ONE 4(11): e7824. doi:10.1371/journal.pone.0007824



Sixty male Sprague±Dawley rats that initially weighed about 150 g were purchased from Japan SLC, Inc. (Shizuoka, Japan) [22].

Male Wistar rats, weighed 250 g to 300 g, were purchased from the Laboratory Animal Center, Chinese Academy of Medical Science [19].

73 6-7-wk-old male BALB/C-nu/nu mice (weight 18-22 g) were obtained from Shanghai Tumour Institute [No. SCXK (Shanghai) 2002-0001; Shanghai, China [31].

- Randomisation and blinding. The aim of random is to ensure that, as far as possible, any differences in outcome measures observed between the groups can be ascribed purely to the experimental procedures (see footnote 4). Random selection is not the same as haphazard selection; a systematic, physical approach such as tossing a coin or using a table of random numbers or a computer to pick numbers randomly, is necessary for this process. On the other hand, Blinding, where the researcher does not know which treatment the animal has received when judging an experimental result, is an effective way of minimising this bias.

The 61 rats with oral tumours were divided into 4 groups by stratified random sampling [9].

Mice were randomized into four experimental groups [23].

Animals were randomly divided into three groups of 20 rats each [22].

The mice were randomly divided into 3 groups, one control and the two representing experimental conditions [31]

Ten SD rats (aged 10-12 wk and weighing  $280 \pm 30$  g) were randomly divided into five groups: the negative group (NS group), the positive group (CTX group) and three *Gecko* groups [15] (*Gekko gecko* is an animal used as a valued traditional Chinese medicine; in this case the CHM includes non-botanical components).

The evaluation standard of clinical curative effects was drawn up according to the 2nd version of "Clinical Research Guiding Principle of New Medicine of TCM" and other corresponding reports [11].

Blinding, where the researcher does not know which treatment the animal has received when judging an experimental result, is an effective way of minimising this bias

Double blind method was applied in this experiment [14].

#### - Euthanasia procedures

Mice were anesthetized with isoflurane (Abbott Laboratories, Botany, NSW, Australia) and placed in a supine position, and the prostate was exposed by laparotomy [23].

Animals were euthanized under general anaesthesia (overdose of pentobarbital) at the termination of each study. This method is consistent with the recommendation by the Panel of Euthanasia of the American Veterinary Medical Association [16].

Rats were sacrificed with halothane 3 weeks after tumour inoculation, and their lungs were removed and placed for 24 h in Bouin solution 72% saturated picric acid solution, 23% formaldehyde 37% solution and 5% glacial acetic acid [3].

Mice with tumours were killed through neck vertebrae dislocation [28].

The mice bearing S180 were killed by dislocation of cervical vertebra 3 wk after treatment [29].

- Sample size. It is argued that tight control of experimental conditions minimises variance and allows for small sample sizes, but most animal studies are still hopelessly underpowered. Systematic review has allowed the analysis of sample size in studies of FK506 in animal models

of stroke; the observed variance suggests that 65 animals per group would be needed to give an 80% chance of detecting an improvement in outcome of 20%<sup>5</sup>

Eighty Sprague-Dawley (SD) male rats, aged 6–7 weeks, were fed 4NQO in their drinking water for 36 weeks. [...]. Another 20 SD rats received pure water (control group). [...] 61 surviving rats. The 61 rats with oral tumours were divided into 4 groups [9].

After a 5 d acclimatization period, 165 rats were assigned into group I (carcinogen-exposed control) and group II (Ganfujian granule treatment). Fresh sterile water was used to prepare DEN solution of 100 mg/mL concentration, to which the rats had free access [18].

#### - Ethical approval granted?

Experimental Animal Administrative Committee of Shanghai granted ethical approval for the animal experiments [9].

The animal study was approved by the Animal Care Committee of North Carolina Central University [27].

All experiments were carried out according to the protocols approved by the Animal Care Committee of the Animal Center at the Chinese Academy of Sciences in Shanghai and in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals [20].

Six-to-eight week- old female DBA/2 mice were purchased from the Jackson Laboratory (Indianapolis, IN) and housed in the LSUHSC-S Animal Resource Facility (four in one cage in microisolators) under standard regulations. The LSUHSC-S Animal Facility is AAALAC approved and maintains a consultation team of two veterinarians. The program is also monitored by the National Institute of Health Office for Protection from Research Risk and the U.S. Department of Agriculture [16].

[...] according to the method described in “National Requirements for *in vivo* Screening of Anti-neoplastic Drugs [29]

This study was conducted in accordance with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University [10].

#### - Long Term studies

For the long-term study (Experiment 2), animals were housed under the same conditions as described above. Except for 30 hamsters serving as negative control (Group A), the remaining 86 animals were topically treated with 0.5% DMBA in 100  $\mu$ l of mineral oil three times a week for 6 weeks. The animals were then randomly divided into three groups with Group B receiving no further treatment (30 animals). Group C and D (28 animal each) were treated with 50 or 100  $\mu$ l Zylamend, respectively, three times per week for another 18 weeks [27].

The QHF formula components and the optimal dose ratio obtained above were used for a verification test. Mice bearing both solid and ascitic tumours were randomly divided into 6 groups: NS, QHF formula, Cinobufotalin (800 mg/kg), Ginsenosides Rg3 (14 mg/kg), PNS (5.5 mg/kg), and Lentinan groups (100 mg/kg). The drugs were diluted to the required concentration with NS and 0.4 ml administered ig once a day for 10 days. The tumour growth inhibition and survival were assayed the same as above [21].

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<sup>5</sup> Macleod MR, O'Collins T, Horky LL, Howells DW, Donnan GA. Systematic review and metaanalysis of the efficacy of FK506 in experimental stroke. *J Cereb Blood Flow Metab.* 2005 Jun;25(6):713-21.

## 6. REFERENCES

This is the list of 31 scientific articles analyzed in the Results section. In these references an asterisk (\*) means that in the research was used a commercially available proprietary product

- [1] Cao Y, Xia QH, Meng H and Zhong AP. Antitumour and synergistic effect of Chinese medicine “Bushen huayu jiedu recipe” and chemotherapy on transplanted animal hepatocarcinoma. *World J Gastroenterol* 11:5218-5220, 2005
- [2]\* Duan YW, Huang Y, Cai LQ, Duan Q and Zhu YS. Inhibition of tumour growth and tumour metastasis by a Chinese herbal formula - ZYD88, in an animal model with metastatic Lewis lung carcinoma. *Oncol Re* 17:1391-1397, 2007
- [3] Hong-Fen L, Waisman T, Maimon Y, Shakhar K, Rosenne E and Ben-Eliyahu S. The effects of a Chinese herb formula, anti-cancer number one (ACNO), on NK cell activity and tumour metastasis in rats. *Int Immunopharmacol* 1:1947–1956, 2001
- [4] Hsu YL, Yen MH, Kuo PL, Cho CY, Huang YT, Tseng CJ, Lee JP and Lin CC. San-Zhong-Kui-Jian-Tang. Traditional Chinese Medicine Prescription, Inhibits the Proliferation of Human Breast Cancer Cell by Blocking Cell Cycle Progression and Inducing Apoptosis. *Biol. Pharm. Bull.* 29: 2388—2394, 2006
- [5]\* Huang YP, Du J, Hong ZF, Chen ZQ, Wu JF and Zhao JY Effects of Kangquan Recipe (康泉方) on Sex Steroids and Cell Proliferation in Rats with Benign Prostatic Hyperplasia. *Chin J Integr Med* 15:289-292, 2009
- [6] Ji DB, Ye J, Jiang YM and Qian BW .Anti-tumour effect of Liqui, a traditional Chinese medicine prescription, in tumour bearing mice. *BMC Complem and Altern Med* 9:20 doi:10.1186/1472-6882-9-20, 2009
- [7] Ji YB, Gao SY and Cheng WP. Effect of Haimiding on the functioning of red cell membrane of FC and H22 tumour-bearing mice. *World J Gastroenterol* 11:823-830, 2005
- [8]\* Ji YB, Gao SY, Ji HR, Kong Q, Zhang XJ and Yang BF. Anti-neoplastic efficacy of Haimiding on gastric carcinoma and its mechanisms. *World J Gastroenterol* 10:484-490, 2004
- [9] Jiang C, Ye D, Qiu W, Zhang X, Zhang Z, He D, Zhang P and Chen W. Response of lymphocyte subsets and cytokines to Shenyang prescription in Sprague-Dawley rats with tongue squamous cell carcinomas induced by 4NQO. *BMC Cancer* 7:40-45, 2007
- [10] Lee Sj, Saiki I, Hayakawa Y, Nunome S, Yamada H and Kim SH. Antimetastatic and immunomodulating properties of a new herbal prescription, Bojung-bangam-tang. *Int Immunopharmacol* 3:147–157, 2003
- [11] Li HZ, Wang H, Wang GQ, Liu J, Zhao SM, Chen J, Song QW, Gao W, Qi XZ and Gao Q. Treatment of gastric precancerous lesions with Weiansan. *World J Gastroenterol* 12:5389-5392, 2006
- [12] Li X, Shi ZM, Feng P, Wen ZY and Wang XJ. Effect of Qi-protecting powder (Huqi San) on expression of c-jun, c-fos and c-myc in diethylnitrosamine-mediated hepatocarcinogenesis. *World J Gastroenterol* 13:4192-4198, 2007
- [13] Lin LW, Sun Y, He YM, Gao SD, Xue ES, Lin XD, Yu LY, Lin XF and Yang YH. Percutaneous intratumoural injection of traditional Chinese herbal compound medicine Star-99 in treatment of hepatocellular carcinoma of mice. *Hepatobiliary Pancreat Dis Int* 2004; 3:9: 49-54.

- [14] Lin LW, Lin XD, He YM, Gao SD and Xue ES. Experimental study on ultrasound-guided intratumoural injection of “Star-99” in treatment of hepatocellular carcinoma of nude mice. *World J Gastroenterol* 9:701-705, 2003
- [15] Liu F, Wang JG, Wang SY, Li YL, Wu YP and Xi SM. Antitumour effect and mechanism of Gecko on human esophageal carcinoma cell lines in vitro and xenografted sarcoma 180 in Kunming mice. *World J Gastroenterol* 14:3990-3996, 2008
- [16] Liu J, Gu X, Robbins D, Li G, Shi R, McCord JM and Zhao Y. Protandim, a fundamentally new antioxidant approach in chemoprevention using mouse two-stage skin carcinogenesis as a model. *PLoS One*. 2009;4(4):e5284. Epub 2009 Apr 22
- [17] Lu Y and Wu, LQ. Efficacy of intra-tumour injection of Xiao-Zhi-Ling on transplanted hepatoma in rats. *Biol. Pharm. Bull.* 29: 2388—2394, 2006
- [18] Qian Y and Ling C. Preventive effect of Ganfujian granule on experimental hepatocarcinoma in rats. *World J Gastroenterol* 10:755-757, 2004
- [19] Shi XY, Zhao FZ, Dai X, Ma LS, Dong XY and Fang J. Effect of jianpiyiwei capsule on gastric precancerous lesions in rats. *World J Gastroenterol* 8:608-12, 2002
- [20] Sun H, Li TJ, Sun LN, Qiu Y, Huang BB, Yi B and Chen WS. Inhibitory effect of traditional Chinese medicine Zi-Shen Pill on benign prostatic hyperplasia in rats. *J Ethnopharmacol* 115:203–208, 2008
- [21] Tao C, Dan L, Ya-ling F and Wei H. Screening of QHF formula for effective ingredients from Chinese herbs and its anti-hepatic cell cancer effect in combination with chemotherapy. *Chin Med J* 121:363-368, 2008
- [22]\* Tatsuta M, Iishi H, Baba M, Narahara H, Yano H and Sakai N. Suppression by Chai-hu-gui-zhi-tang of the development of liver lesions induced by N-nitrosomorpholine in Sprague±Dawley rats. *Cancer Lett.* 152:31-6, 2006
- [23]2\* Vandyke K, White MY, Nguyen-Khuong T, Ow K, Luk SC, Kingsley EA, Rowe A, Pang SF, Walsh BJ and Russell PJ. Plant-derived MINA-05 inhibits human prostate cancer proliferation in vitro and lymph node spread in vivo. *Neoplasia* 9:322-31, 2007
- [24] Wang L, Zhou GB, Liu P, Song JH, Liang Y, Yan XJ, Xu F, Wang BS, Mao JH, Shen ZX, Chen SJ and Chen Z. Dissection of mechanisms of Chinese medicinal formula Realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia. *Proc Natl Acad Sci USA* 105:4826–4831, 2008
- [25]\* Wu LQ, Lu Y, Lu HJ, Zhao ZG and Yang M. Efficacy of intra-tumour injection of Kang-Lai-Te in treating transplanted hematoma in rats. *Hepatobiliary Pancreat Dis Int.* 3:580-584, 2004.
- [26] Xu Q, Brabham JG, Zhang ZS, Munster P, Fields K and Yu RZH. Chinese herbal formula, Bing de Ling, enhances antitumour effects and ameliorates weight loss induced by 5-fluorouracil in the mouse ct26 tumour model. *Dna and cell biology.* 24:470–475, 2005
- [27]\* Yang P, Sun Z, Chan D, Cartwright CA, Vijjeswarapu M, Ding J, Chen X and Newman RA. Zylflamend reduces LTB4 formation and prevents oral carcinogenesis in a 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster cheek pouch model. *Carcinogenesis* 29:2182–2189, 2008
- [28] You JS, Huang HF and Hau DM. Effects of Xuefu Zhuyu Tang and Mitomycin C on Liver Tumours in Mice. *Chang Gung Med J V* 26: 2003



- [29] Yu ZH, Wei PK, Xu L, Qin ZF and Shi J. Anticancer effect of Jinlongshe granules on in situ - transplanted human MKN-45 gastric cancer in nude mice and xenografted sarcoma 180 in Kunming mice and its mechanism. *World J Gastroenterol* 12: 2890-2894, 2006
- [30] Zhang Z, Wang Y, Yao R, Li J, Yan Y, Regina M, Lemon WL, Grubbs CJ, Lubet RA and You M. Cancer chemopreventive activity of a mixture of Chinese herbs antitumour B) in mouse lung tumour models. *Oncogene* 23:3841–3850, 2004
- [31]\* Zhao AG, Li T, You SF, Zhao HL, Gu Y, Tang LD and Yang JK. Effects of Wei Chang An on expression of multiple genes in human gastric cancer grafted onto nude mice. *World J Gastroenterol* 14: 693-700, 2008