Tentative Fingerprint-Efficacy Study of *Houttuynia cordata* Injection in Quality Control of Traditional Chinese Medicine

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To establish potent fingerprint for quality control of traditional Chinese medicine, *Houttuynia cordata* (Saururaceae) injection (HCI), the attempt on fingerprint-efficacy was developed in this study. HCI from ten different factories were determined by gas chromatography-mass spectrum (GC-MS) and classified by hierarchical clustering. The anti-inflammatory effect of HCI was characterized with the rat pleurisy model induced by carrageenin and the mice ear edema model by xylene. The results showed that anti-inflammatory effect of the injections from most of factories on the two models was significant. There was corresponding relationship between the fingerprint of HCI and efficacy to certain extent. The main common constitutes in injection from the factories that possess anti-inflammatory activity were analysed with GC-MS and identified using the NIST Mass Spectral Database. This common pattern of HCI based on the efficacy was helpful for the purpose of quality control.

Key words *Houttuynia cordata* injection; fingerprint-efficacy; anti-inflammatory; quality control

Traditional Chinese medicine (TCM) is a unique medical system practiced in China and the Far East for thousands of years. In 1985, the World Health Organization estimated that about 80% of the world's population relied on traditional medicines including Traditional Chinese medicines (TCMs) for their primary health care needs.[1)] More and more people are using TCMs to achieve optimum health and to prevent disease conditions than ever before.

Houttuynia cordata Thunb. (Saururaceae) is a commonly used TCM in China and other countries in Asia. It has the functions of relieving fever, resolving toxin, reducing swelling, draining pus and promoting urination.[2—4)] A previous study showed that the steam distillate prepared from fresh plants of *H. cordata* possessed direct inhibitory activity against herpes simplex virus type 1 (HSV-1), influenza virus and human immunodeficiency virvs type 1 (HIV-1) without showing cytotoxicity.[5)] During the period of the Severe Acute Respiratory Syndrome (SARS) outbreak, *H. cordata* was one of the ingredients in the SARS prevention formulas recognized by the Health Ministry of China. Also, *H. cordata* is of high dietary value. It is a wild vegetable with abundant nutrients. *Houttuynia cordata* injection (HCI) was the aqueous solution of the steam distillate from plants of *H. cordata*.

However, as WHO noted in "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines,"[6)] "Despite its existence and continued use over many centuries, and its popularity and extensive use during the last decade, traditional medicine has not been officially recognized in most countries… The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its use worldwide. The reasons for the lack of research data are due not only to health care policies, but also to a lack of adequate or accepted research methodology for evaluating traditional medicine." As a result, fingerprint technique emerged and was accepted as a powerful tool for the evaluation and quality control of multi-component TCMs and their finished products.[7—9)]

H. cordata injection, like many other TCMs liquid injections, was compulsorily carried quality control using fingerprint. It is a pity that the existing fingerprints of HCI focus on naturally occurring constituents and the chemically characteristic ratios of them, and those substances present in fingerprint are not always active constituents that play the leading role in treatment. That is, the chemical profile by itself is insufficient in determining the efficacy of HCI, this kind of fingerprint only improves quality control and standardization, and what on earth brings the effect on disease is still a mystery, which makes modernization and standardization of TCMs sound bizarre and unreliable to the people. So, it is imperative and urgent under this situation to strengthen the study of fingerprint-efficacy, find the relationship between the fingerprint and the efficacy, provide science base for evaluation of HCI, establish integrated evaluation system and finally develop fingerprint containing main active constitutes and representing curative effect.

This study aims to tentatively find corresponding relationship between the fingerprint and efficacy of HCI**,** to provide an innovative way to investigate TCMs with the help of the multivariate statistic analysis, which might provide some insight for the quality control of TCMs.

Experimental

Reagents and Materials Carrageenin was purchased from Sigma Chemical CO., and xylene was purchased from Hunan Normal University. HCI was gift from 10 different factories. Sterile saline injection was obtained from Hunan Zhengqing Pharmaceutical Group Co. LTD, China.

Animals Male Wistar rats weighing 223±40 g and K.M. mice of both sexes weighing 22±2 g were purchased from Animal Center, Central South University, China. All animals were kept at 23±1 °C with a 12 h light/dark cycle. They had free access to water and diet and were acclimatized at least 2 week before starting the experiments. All procedures described were reviewed and approved by the University animal ethical committee.

Sample Preparation and Fingerprint Analysis Two hundred milliliters of injection was introduced in a round bottom flask. Hydrodistillation was carried as stated in appendix XD of Chinese pharmacopoeia, Vol. 1, 2000. The essential oils were collected and dissolved in 2 ml hexane, and then stored in a refrigerator at 4 °C before use.

GC-MS analysis was performed on a Shimadzu GC-2010 (Kyoto, Japan) gas chromatography instrument coupled to a Shimadzu QP2010 mass spectrometer (Compaq-Pro Linear data system, class5k software), equipped with

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an OV-1 capillary column (30 m×0.25 mm I.D., film thickness 0.25 μm). The column was maintained at 50 °C after injection for 6 min, and programmed to 230 °C at the rate of 10 °C/min, then held for 16 min. The temperature of the injection port and interface was set at 280 °C. Ion source: EI (220 °C). Helium was used as the carrier gas at 0.7 ml/min. One microlitre of the sample was injected in the 10 : 1 split mode. The mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70 eV and the scan rate was 5 scan/s. The mass spectrometer was operated with a scan mass range of 20 to 450 atomic mass units. Solvent delay: 6 min. The analytes were identified using the NIST Mass Spectral Database.

Drug Treatment and Induction of Rat Pleurisy The animals were divided into 11 groups randomly, and each group had 12 rats. HCI at 5.4 ml/kg body weight was injected once intravenously everyday and lasted for 6 d. Pleurisy was induced by intra-thoracic injection of 0.1 ml of 2.0% carrageenin, 24 h after the last injection of tested drugs according to a previously described method.[10] Injection of carrageenin was given between the seventh and eighth ribs under the right upper limbs through a gauge needle, with penetration restricted to 6 mm by carefully inserting it through a rubber disc.[11,12] Another injection of treatment drugs was given to animals 3 h after the inflammatory stimulus. Control animals received the irritant and an equal volume of sterile saline. The pleural cavity was opened and rinsed with 1 ml of cold saline containing 20 IU/ml heparin. The fluid was removed by mild suction and its volume was measured. It was evaluated for leucocyte count and protein. A blood aliquot was collected, and white blood cell count (WBC) was determined. All parameters were compared with those of control animals treated with sterile saline injection.

Drug Treatment and Induction of Ear Edema The animals were divided into 11 groups randomly, and each group had 16 mice. The test was performed as previously described.[10,13,14] HCI at 4.0 ml/kg body weight was injected hypodermically near the right ear twice everyday and lasted for 3 d. A total of 20 μl of xylene was applied to the inner and outer surface of the right ear of each mouse 30 min after the last injection of tested drugs. The left ear remained untreated. Control animals received the irritant and an equal volume of sterile saline. The mice were sacrificed by cervical dislocation 40 min later and the plug (9 mm in diameter) was removed with a stainless steel punch from both the treated ear and the untreated ear. The difference in weight between the two plugs was taken as a measure of edematous response.

Measurement of Total Volume of Pleural Fluid, Total Leucocyte Count in Pleural Fluid The total volume of the fluid collected from the pleural cavity was measured. The 20 μl of pleural fluid was diluted with 0.38 ml diluent (distilled water containing 1.5% acetic acid and 0.01% gentian-violet) and total leucocyte count (TLC) was measured using a SB-K-25 chamber under a light microscope.

Measurement of Total Protein in Pleural Fluid The fluid collected from the pleural cavity was centrifuged (3000 rpm for 5 min) and protein concentration in the supernatant was assayed by the Coomassie brilliant blue method because of its high sensitivity and convenience.[15,16]

Measurement of White Blood Cell Count (WBC) A blood aliquot was collected, and WBC was determined with a Coulter counter (Coulter STKS, Hialeah, FL, U.S.A.).

Extent of the Edema and Percentage of Inhibition These plugs cut from mice were weighed on an electronic balance, and the extent of the edema was expressed as the difference in the weight of plugs from inflamed and untreated ears.[17,18] The percentage of inhibition was calculated as follow,

$$\text{percentage of inhibition} = \frac{a-b}{a} \times 100\% \quad (1)$$

where 'a' is the mean edema extent of controlled mice, 'b' is the mean edema extent of treated mice with HCI.

Statistical Analysis Effect values are expressed as mean±S.E.M. They were further analysed using one-way analysis of variance (ANOVA) test to calculate significance of results. $p<0.05$ was considered as indication of significance. Clustering analysis of fingerprint was based on Hierarchical clustering using Euclidean distance.

Results and Discussion

GC-MS Fingerprint Analysis and Clustering of HCI Chemical fingerprints obtained by hyphenated chromatographies, are strongly recommended for the purpose of quality control of TCMs, since they might represent appropriately the "chemical integrities" of TCMs and therefore be used for authentication and identification of TCMs. Based on the conception of phytoequivalence, chromatographic fingerprint of a TCM is, in practice, a chromatographic pattern of the extract of some common chemical components of pharmacologically active and or chemically characteristics. In order to achieve a stable and reproducible chromatographic fingerprint of HCI, a comprehensive method validation on the developed GC-MS fingerprint analysis was conducted. All results indicate that the developed methodology is applicable for establishing a GC-MS fingerprint of HCI. The detailed results can see our previous paper.[19] Samples of HCI were analyzed under the established GC-MS conditions (see sample preparation and fingerprint analysis section above). Table 1 shows the factories that provide HCI. The profiles of HCI from 10 different factories are presented in Fig. 1. From Fig. 1, it is found that there are some fingerprint differences among samples from different factories. However, it is difficult to explain clearly fingerprint differences among samples concretely. So clustering in statistics was introduced to deal with this problem.

Table 1. Factories and Batch Number of HCI

Factory No.	Factory	Batch No.
1	Sanai	040401
2	Xingzhong	20040306
3	Huanghe	04050070
4	Qingchunbao	0405051
5	Sanjiu	040601
6	Zhengqin	0309413
7	Yusi	04072101
8	Shenghe	041203
9	Shuanghe	040201
10	Wanrong	041226-2

Clustering is the art of grouping together pattern vectors that in some sense belong together because of similar characteristics. It provides both a visual representation of complex data and a method for measuring similarity between experiments. The hierarchical clustering is now presented using the Euclidean distance as the distance between pattern vectors, and the average distance between clusters as distance between clusters. The complete process of hierarchical clustering analysis on all fingerprints using the statistics functions can be summarized as follows:

(1) Find the Similarity or Dissimilarity between Every Pair of Fingerprints of HCI from Different Factories: In this case, calculate the 'distance' between fingerprints using the Euclidean distance function, the most common distance function between clusters. It is defined on a given vector space as:

$$d(x,y) = \sqrt{(x-y)^T(x-y)} = \left(\sum_{i=1}^{n}(x_i-y_i)^2\right)^{1/2} \quad (2)$$

where x and y were two fingerprints of HCI from different factories, and x_i, y_i were the ith elements in x and y respectively and n was the number of the elements in the fingerprints. Here, d calculated was closer to 0, the two fingerprints

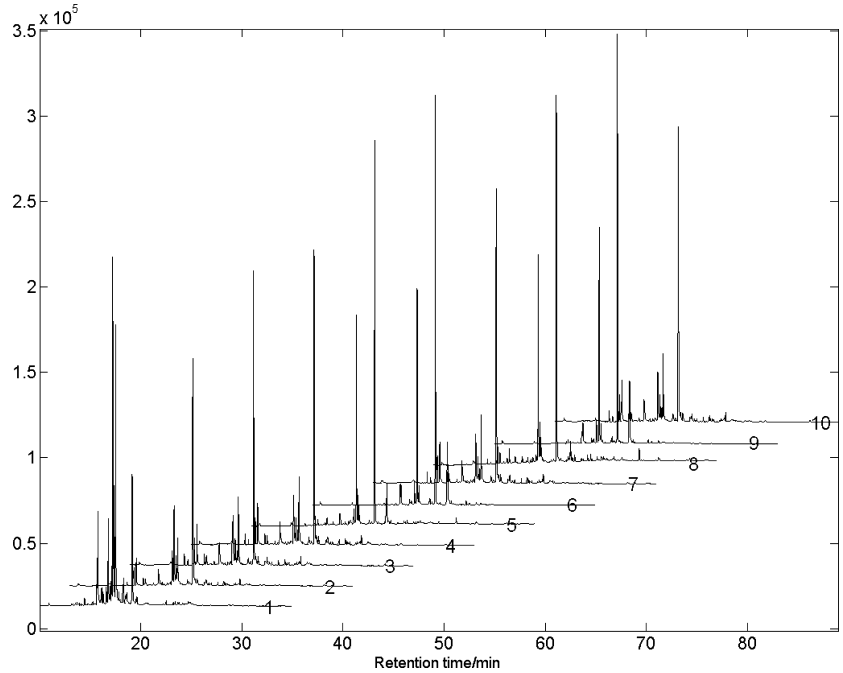

Fig. 1. Chromatographic Fingerprints of HCI from Different Factories

1, Sanai; 2, Xingzhong; 3, Huanghe; 4, Qingchunbao; 5, Sanjiu; 6, Zhengqing; 7, Yusi; 8, Shenghe; 9, Shuanghe; 10, Wanrong. Chromatographic conditions: an OV-1 capillary column (30 m×0.25 mm I.D., film thickness 0.25 μm); the column was maintained at 50 °C after injection for 6 min, and programmed to 230 °C at a rate of 10 °C/min, then held for 16 min; the temperature of the injection port and interface was set at 280 °C; ion source: EI (220 °C); helium was used as the carrier gas at 0.7 ml/min; one microlitre of the sample was injected in the 10 : 1 split mode; the mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70 eV and the scan rate was 5 scan/s.

were more similar.

(2) Fuse the Two Most Similar Fingerprints and Recalculate the Distances: When hierarchical clustering algorithm merges two fingerprints to generate a new cluster, it should calculate the distances between the new cluster and remaining fingerprints. There are many different ways to do that, and we call them 'linkage method.' In this case, average linkage was introduced. Let C_i be a new cluster, C_j be a remaining cluster. Average linkage is defined as follows:

$$d(C_i,C_j)=\frac{1}{n_{C_i}n_{C_j}}\sum_{x\in C_i,y\in C_j}d(x,y) \quad (3)$$

It is the average of all pairwise distances between fingerprints in the two clusters. Here, n_{C_i} and n_{C_j} were the number of fingerprints in clusters C_i and C_j respectively.

(3) Repeat Step 2 until All Fingerprints Aare in One Cluster: As fingerprints are paired into binary clusters, the newly formed clusters are grouped into larger clusters until a hierarchical tree is formed.

The clustering result is shown in Fig. 2. Looking at the endrogram of the fingerprints in Fig. 2, an obvious cluster is seen. Depending on the distance the samples could be classified into four groups: (1) Zhengqing, Shuanghe, Sanjiu and Yusi; (2) Qingchunbao, Shenghe and Xingzhong; (3) Wanrong and Huanghe; (4) Sanai.

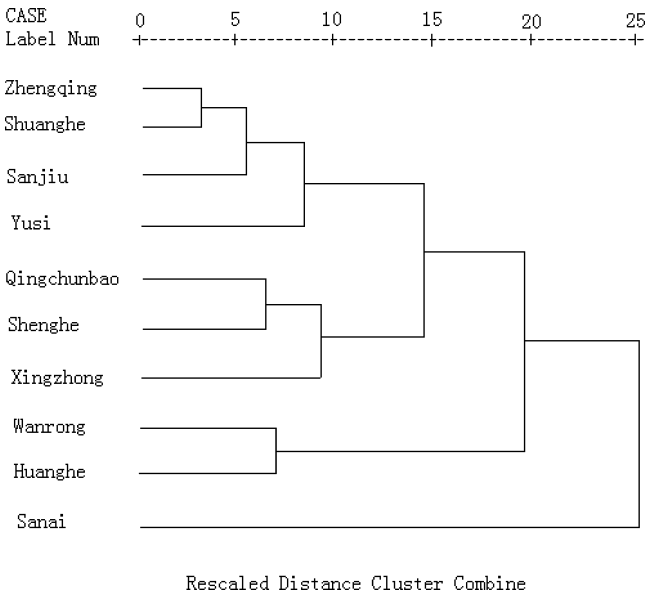

Fig. 2. Dendrogram Showing the Hierarchical Clustering Results for the Fingerprints of HCI from 10 Factories, Using Euclidean Distance and Aaverage Linkage

Anti-inflammatory Effect of HCI on Xylene Induced Ear Edema Topical anti-inflammatory activity of HCI was evaluated using inhibition of the xylene-induced ear edema in mice. Since the animals were treated with HCI before ear edema was induced, to exclude the possible case that ear edema has been induced by injection of HCI, in the primary test, two groups of mice were examined before and after injection of HCI respectively. The result showed that there was no weight difference between ears before treatment with HCI and those after treatment, or no difference between left and right ear after treatment with HCI.

Previous paper also reported that the xylene-induced mouse ear edema method has certain advantages for natural product testing and has a good predictive value for screening anti-inflammatory agents.[20] Topical application of xylene induced cutaneous inflammation at the ears of mice and caused

significant increase in ear plug weight of the right ear when compared to the left ear. The experiment was conducted as experimental section stated above. The difference in weight between the two plugs was taken as a measure of edematous response. The change of the difference administrated by HCI from different factories is shown in Table 2.

After ANOVA test, the result in Table 2 shows that HCI from factories Xingzhong, Huanghe, Qingchunbao, Sanjiu, Zhengqing, Yusi, Shenghe, Shuanghe and Wanrong have significant anti-inflammatory activity on xylene induced ear edema, compared with effect of sterile saline, while factory Sanai shows no significance.

Anti-inflammatory Effect of HCI on Carrageenin Induced Pleurisy Pleurisy induced by carrageenin is a good acute inflammatory model characterized by protein rich fluid accumulation including albumin, globin and fibrinogen so on, and leucocyte infiltration in the pleural cavity. In the same way, to exclude the possible case that pleurisy has been induced by injection of HCI, two groups of rats were treated. All parameters showed no difference between rats before treatment with HCI and those after treatment.

After ANOVA test, the result in Table 3 shows anti-inflammatory effect of HCI on carrageenin induced pleurisy. The result shows that HCI from all factories produces significant ($p<0.05$) reduction of indices in carrageenin induced pleurisy when compared to the control group, except that from Sanai shows no significance in index of total cells. That is, in despite of difference in efficacy, HCI from all factories is potently effective in curbing inflammatory model induced by carrageenin, which should be the result of HCI decreasing the capillary permeability.

Fingerprint-Efficacy Analysis To find the corresponding relationship between components in the fingerprint of HCI and efficacy, regression will be done usually. But owing to severe non-linearity between them, multivariate statistic analysis, clustering analysis, was used to help explain the relationship here. Clustering analysis of chromatographic fingerprints of HCI from 10 factories was done. The result is shown in Fig. 2. As stated above, the samples could be classified into four groups visually. However, only two kinds of result were defined in anti-inflammatory test, one with significant anti-inflammatory activity, the other with no significance, hence we reconstruct the dendrogram into two groups. Factory Sanai alone falls into a cluster and other nine factories fall into the other cluster, because group (1) and (2) and (3) are more similar to each other than to group (4). That is to say, factory Sanai alone falls into a cluster and other nine factories fall into the other cluster.

From Fig. 1, a specific point that distinguishes one cluster showing anti-inflammatory activity from the other cluster showing no anti-inflammatory activity was that the content of methyl *n*-nonyl ketone and bornyl acetate (retention time, 19.0—19.3 min) were the highest in the fingerprint of the cluster that showed significant anti-inflammatory activity, while the content of 1-nonanol, 4-terpineol, α-terpineol and *n*-decanal (retention time, 17.0—17.6 min) were the highest in the cluster that showed no significance. In fact, there are also some differences among the fingerprints of HCI from Xingzhong, Huanghe, Qingchunbao, Sanjiu Zhengqing, Yusi, Shenghe, Shuanghe and Wanrong that fall into a cluster (Fig. 1).

The difference in concentration of the components of HCI, especially the variation in quantities of major and/or minor components might be responsible for the different anti-inflammatory activity. It may be due to the synergy or sum total of all the constituents according to Chinese medicine theory, even like a popular statement in this connection, that is $1+1>2$. The component combination in HCI of the cluster with anti-inflammatory activity was considered to be better than those of the cluster with anti-inflammatory activity.

From the results obtained by these two models, HCI from all factories is potently effective in curbing inflammation though some differences exist, except that from the factory Sanai is of no significance in ear edema induced by xylene, which suggests there are some "sameness" among all the in-

Table 2. Anti-inflammatory Effect of HCI on Xylene Induced Ear Edema

Group		Extent of the edema (mg) Mean±s	Percentage of inhibition (%)
Sterile saline		11.2±5.5	—
HCI	Sanai	7.4±5.0	33.4
	Xingzhong	6.9±4.7*	37.9*
	Huanghe	6.2±4.1*	44.6*
	Qingchunbao	5.7±4.1*	48.4*
	Sanjiu	4.7±3.4*	57.8*
	Zhengqing	4.7±3.3*	57.3*
	Yusi	5.0±3.6*	55.3*
	Shenghe	5.3±3.5*	52.5*
	Shuanghe	5.6±3.8*	50.1*
	Wanrong	6.3±4.2*	43.7*

Data represent the mean of difference in ear weight (mg)±S.E.M., ($n=16$). $*p<0.05$, significant as compared to the control.

Table 3. Anti-inflammatory Effect of HCI on Carrageenin Induced Pleurisy

Group		Total volume (ml) Mean±s	Total protein (mg/ml) Mean±s	TLC ($\times10^6$/ml) Mean±s	WBC Mean±s ($\times10^9$/l)
Sterile saline		1.12±0.41	26.18±17.17	65.79±32.24	21.66±5.17
HCI	Sanai	0.22±0.15*	5.47±5.35*	49.60±28.01	10.98±4.03*
	Xingzhong	0.10±0.05*	2.03±1.46*	36.87±23.00*	11.52±2.28*
	Huanghe	0.20±0.13*	5.32±5.40*	47.82±18.37*	11.58±2.42*
	Qingchunbao	0.09±0.02*	4.00±1.67*	37.74±16.35*	11.45±4.29*
	Sanjiu	0.08±0.03*	5.24±1.78*	48.08±15.65*	10.85±4.94*
	Zhengqing	0.16±0.09*	3.35±2.18*	29.98±10.88*	10.43±1.84*
	Yusi	0.18±0.10*	3.02±2.11*	28.71±12.69*	10.93±2.54*
	Shenghe	0.12±0.06*	4.8±1.28*	46.11±14.76*	10.63±3.87*
	Shuanghe	0.13±0.04*	4.50±2.87*	39.24±19.12*	11.24±5.01*
	Wanrong	0.15±0.11*	5.32±3.61*	46.82±20.62*	10.67±3.25*

Each value represents mean±S.E.M. ($n=12$). $*p<0.05$ significant as compared to the control.

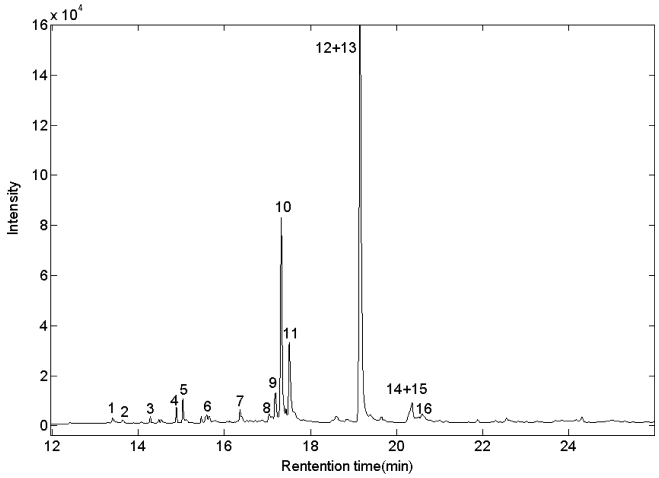

Fig. 3. Common Pattern of HCI from 9 Factories That Possess Anti-inflammatory Activity

1, β-pinene; 2, β-myrcene; 3, α-pinene; 4, β-cis-ocimene; 5, γ-terpinene; 6, β-linalool; 7, Z-β-terpineol; 8, 1-nonanol; 9, 4-terpineol; 10, α-terpineol; 11, *n*-decanal; 12, methyl *n*-nonyl ketone; 13, bornyl acetate; 14, *n*-decanoic acid; 15, 1-decen-3-one; 16, acetic acid geraniol ester.

jection from those factories possessing anti-inflammatory activity.

By definition, a chromatographic fingerprint of a TCM is, in practice, a chromatographic pattern of the extract of some common chemical components of pharmacologically and/or chemically active characteristics.[9] This chromatographic profile should be featured by the fundamental attributions of "integrity" and "fuzziness" or "sameness" and "differences" so as to represent the TCMs investigated.

Presently, to find "sameness" of the fingerprint of HCI from those factories, the rule accepted that calculate "sameness" is to evaluate the mean of all fingerprints. We call the mean of all fingerprints as common pattern of them. The mean of all fingerprints was expressed by the following formulae:

$$\bar{x} = \frac{\sum x_i}{n} \quad i=1, 2, 3, \ldots n \tag{4}$$

where x_i was the *i*th fingerprint, and *n* was the number of all fingerprints.

In the present study, because HCI from the factory Sanai showed no significance in ear edema induced by xylene, the calculating of "sameness" used only the fingerprint of HCI from 9 factories that showed activity in two anti-inflammatory models. The result obtained is shown in Fig. 3.

We know that the things that really act on disease should be substantial chemical constitutes. These activities may be attributed to the presence of compounds found in all HCI. The chemical components were complimentary to each other and performed better as a whole. The common pattern obtained through calculating the mean of all fingerprints possessed "integrity" and "fuzziness". To elaborate on "sameness" and determine the presence of the same components among the chromatographic fingerprints of HCI from 9 factories that possess anti-inflammatory activity, GC-MS technique and the NIST Mass Spectral Database are adopted to analyze and identify each component. Many components appeared in all GC-MS profiles of HCI from 9 factories simultaneously. Several main commom components obtained in GC-MS fingerprint were methyl *n*-nonyl ketone, bornyl acetate, α-pinene, β-pinene, β-linalool, 1-nonanol, 4-terpineol, α-terpineol, *n*-decanal, *n*-decanoic acid, 1-decen-3-one and acetic acid geraniol ester so on (Fig. 3). The peak area of those components comparative to total peak area is over eighty-five percent.

As a whole, this common pattern of HCI based on the efficacy is more reasonable and acceptable than pure chemical fingerprint standard, so it could be suggested to help carryout the quality control of HCI.

Of course, to evaluate reasonably the relationship between the efficacy and the chemical fingerprint of TCMs is not a trivial task. HCI like other traditional Chinese medicines have a great deal of efficacy on different disease. The present study on the inflammation is just an attempt, which might provide some insight for the quality control of herbal medicines, but is far from sufficient to meet the criteria needed. There is a long way to go for the fingerprint-efficacy study.

Conclusions

HCI from 10 different factories were determined by gas chromatography-mass spectrum and classified by hierarchical clustering. The injection from factory Sanai alone was merged into a cluster and those of the other nine factories were merged into the other cluster. The anti-inflammatory activity of HCI was characterized through the rat pleurisy model induced by carrageenin and the mice ear edema model by xylene. The result showed that anti-inflammatory effect of the injections from 9 factories on the two models was significant. The sameness of the injections from 9 factories that possess anti-inflammatory activity was calculated, and the main common constitutes were identified using the NIST Mass Spectral Database. This common pattern of HCI based on the efficacy was suggested to help carry out the quality control of HCI.

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