Acute and Subacute Toxicity of the Extract of *Aristolochiae Fructus* and Honey-Fried *Aristolochiae Fructus* in Rodents

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Received September 19, 2013; accepted December 20, 2013; advance publication released online December 26, 2013

*Aristolochiae Fructus* (AF) and honey-fried *Aristolochiae Fructus* (HAF) have been used in China for thousands of years as an anti-tussive and expectorant drug. Few clinical cases were reported associated with the toxicity of AF and HAF, although relatively high contents of aristolochic acids (AAs) were found in them. This work was designed to compare the acute and subacute toxicity of AF and HAF in order to provide references for safe clinical use and to evaluate the possibility of reducing toxicity of AF by honey-processing. The extracts of the herb were fed to mice or rats via gastric tube. Various toxic signs and symptoms, body weights, serum biochemical assay, organ weights and histopathology were used to evaluate the toxic effects. The median lethal dose (LD50) of AF and HAF are 34.1±7.2 g/kg/d and 62.6±8.0 g/kg/d with a 95% average trustable probability (p=0.95), respectively. The subacute results showed a dose-dependant relationship of the toxicity of AF and HAF. Even in the high dose groups, only moderate toxicity was observed. Honey-frying and decoction with water can decrease the contents of AAs, and attenuate the toxic effects of AF. But sufficient attention should be still paid to the safety of AF and HAF due to the existence of AAs.

**Key words** acute toxicity; subacute toxicity; *Aristolochiae Fructus*; honey-fried *Aristolochiae Fructus*; honey-frying technology; herbal safety

*Aristolochiae Fructus* (AF), the dry-ripe fruit of *Aristolochia contorta* Bge. or *Aristolochia debilis* Sieb. et Zucc., has been used in China for thousands of years as an anti-tussive and expectorant drug.1 Honey-fried AF (HAF) has higher frequency of use than AF in clinic.

The herbs and herbal remedies containing aristolochic acids (AAs) have drawn extensive attention because they are associated with the development of a chronic, progressive renal disease, designated as aristolochic acid nephropathy (AAN).2 AAN case was reported initially in a group of women in Belgium who developed severe renal disease after ingesting slimming pills containing *Aristolochia fangchi*3. Now, AAs have been proven to be nephrotoxic,4–6 carcinogenic6–8 and mutagenic.9–9 Therefore, most AAs-generating herbs and herbal preparations have been banned in many countries, including China. Some literatures10–15 have reported that AAs contents in herbs were in the following order: *A. manshuriensis* > *A. fangchi* > *A. Ridix* > *A. Fructus* (AF) > *A. Herbra*. However, few clinical cases were reported to be associated with the toxicity of AF, AF and HAF were still listed in Chinese Pharmacopoeia (CP).1 For safe clinical use, it is quite necessary to evaluate the toxicity of AF and HAF since relatively high contents of AAs are found in them. Up to now, the pharmacological and toxicological actions of AAs,3,4,6,16–20 *A. manshuriensis*,21–23 and *A. fangchi*24–26 were well known, but only one piece of literature could be accessed on the sub-chronic toxicity of the aqueous extract of AF27.

Drug processing is a traditional pharmaceutical technology in China, and plays an important role in reducing the toxicity of the traditional drugs. In preliminary study, we have found that processing technology can reduce the contents of AAs in AF,28 which indicates that the toxicity of AF could be attenuated with drug processing from chemical viewpoint. This work presents the acute and subacute toxicity of AF and HAF in rodents based on classical toxicological methods. We hope the study can provide references for their clinical use, and help to validate the feasibility of detoxification by honey-frying technology.

**MATERIALS AND METHODS**

**Instruments and Apparatus** Histopathological examination was performed with an inverted phase contrast Olympus CX41RF microscope (Olympus Co., Tokyo, Japan) outfitted with a digital camera. Biochemical analysis was carried out with a UV-3000 spectrophotometer (Shimadzu, Kyoto, Japan). Chromatographic analysis was performed with an Agilent 1260 LC system (CA, U.S.A.), equipped with a quaternary pump, a degasser, and a column compartment. A RE-5250 rotary evaporator (Shanghai Yarong Biochemistry Apparatus Co., Ltd., Shanghai, China) was used to concentrate the aqueous extracts of AF and HAF.

**Collection and Extraction of AF and HAF** AF was purchased from Haixing Chinese Herbal Pieces Co., Ltd. (Bozhou, Anhui, China), and was authenticated as the fruits of *Aristolochia contorta* Bunge by Professor Wuliang Yang (Jiangxi University of Traditional Chinese Medicine, JXUTCM). Voucher specimens are preserved in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, JXUTCM, Jiangxi, China.

Part of AF was processed with honey to obtain HAF in our lab according to the literatures.1,2 Honey (30 g) was cooked with water to suitable viscoscity, and mixed well with 100 g of AF, then kept for 0.5 h at 80°C. Afterwards the mixtures were fried with slow fire till the AF darkened and drug perfume suffused all around. Then the mixtures were cooled to room

The authors declare no conflict of interest.

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temperature, and weighed. The dried AF and HAF were ground to pass 20 mesh filters. The ground herbs were refluxed with water twice (2 h each), condensed to fluid extract, and stored in the fridge before use. A range of solutions were diluted with distilled water to make up required concentrations, respectively.

**Analysis of the Extracts** The four main aristolochic acids (Fig. 1) in the extracts of AF and HAF were determined by an improved high-performance liquid chromatography method (HPLC). Separation was performed on an Elite Hypersil C18 Column (5 μm, 200×4.6 mm). The mobile phase was composed of methanol (A) and 1% formic acid (B). The gradient procedures were as follows: 0–20 min, 20–45 A%; 20–30 min, 45–55 A%; 30–45 min, 55–70 A%; 45–50 min, 70–100 A%. The post-run time was 10 min to balance the column. The other analysis conditions were as follows: column temperature, 30°C; flow rate, 1.0 mL/min; injection volume, 20 μL; detection wavelength, 254 nm.

The fluid extracts (1.0 g) of AF and HAF were weighed accurately, and resolved in 50.0 mL methanol. The solution (1.0 mL) was transferred into a 10-mL volumetric flask, and diluted with methanol to the volume. Samples were filtered through 0.45 μm membrane prior to HPLC analysis.

**Animals and Maintenance** KM mice (Chinese Kunming mice, Certificate No. SCXK (Jiangxi) 2011-001) of each gender and body weight of 17–23 g were provided by the Experimental Animal Center of Jiangxi University of Traditional Chinese Medicine. Sprague Dawley (SD) rats (Certificate No. SCXK (Hunan) 2011–001) of both sexes, and having a body weight of 200±10 g were purchased from Hunan Silaike Jingda Experimental Animal Co., Ltd. (Changsha, China). The animals were maintained in environmentally controlled rooms at 23±3°C under a relative humidity of 50±10% with a 12/12 h light-dark cycle. The rooms were regularly disinfected by UV light. All animals were acclimatized to their new surroundings for 1 week prior to experimental procedures, and were supplied water ad libitum throughout the duration of the experiment. The animals were deprived of food for 12 h before oral administration of the extracts. The University Animal Care and Welfare Committee approved all animal protocols following the Chinese Specifications for the Production, Care and Use of the Laboratory Animals.

**Acute Toxicity** The study was carried out in two phases according to the method. In the preliminary experiment, twenty KM mice were divided into 5 groups (4 mice each group, 2 males and 2 females). In order to find out 0–100% mortality doses range, five large spacing doses of AF extract were orally administrated to each group, respectively. The mice were then kept under the same conditions and continuously observed for general behavior, signs of toxicity and mortality for the first critical 4 h and thereafter daily for 7 d. The same preliminary experiments were carried out for HAF extract.

In the second phase, the animals were divided into 5 groups (10 mice each group, 5 males and 5 females). Each group was segregated according to gender and housed in two plastic cages, 5 mice in one cage. Five doses were selected as the geometric progression mean of 0–100% mortality doses obtained in the preliminary phase. For AF, the oral doses equivalent to raw herb were 84.0, 58.8, 41.2, 28.8 and 20.2 g/kg/d, respectively. For HAF, the doses were 100.0, 80.0, 64.0, 51.2 and 41.0 g/kg/d, respectively. The following procedures, such as behavior observation and mortality record, were same with the preliminary phase. The median lethal doses (LD50) of AF and HAF orally administrated in mice was calculated with improved Kow’s method.

**Subacute Toxicity** Healthy SD rats were randomly divided into 7 groups of 16 (8 males and 8 females), and housed in plastic cages (eight in a cage, segregated by gender). The oral doses of AF extract were calculated according to the obtained median lethal dose. For comparison, the similar doses were adopted for HAF extract. The animal groupings and oral doses are shown in Table 1.

The clinical signs of the rats were observed daily for physiological and behavioral changes. The general symptoms of toxicity and mortality were also monitored once a day. All rats were weighed twice a week during the period of treatment (28 d). Changes of body weight were also recorded and calculated. Twelve-hour urine samples were individually collected in a metabolic cage at one-week intervals.

On the 28th day, all surviving rats were fasted overnight, and sacrificed afterwards for blood collection from femoral artery for biochemical examinations. The non-heparinized blood was allowed to coagulate before being centrifuged (4000 rpm, 10 min) and the serum was separated. Serum assays including serum creatinine (SCr) and blood urea nitrogen (BUN) were performed with standard clinical laboratory kits (Jiancheng Bioengineering Institute, Nanjing, China) by spectrometry.

Immediately after collecting the blood samples, the livers and kidneys were removed and weighed. The relative organ body weight (ROW) ratio of each rat was calculated. The kidneys were then fixed in 10% formalin solution. After organization cropping, paraffin embedding, conventional section,
hematoxylin–eosin (H&E) staining, gradient dehydration, hy-
alizing and sealing film, the pathological investigation of the rat’s renal tissue was performed under the light microscope (×100, ×200, and ×400).

The cortical tissues were then semi-quantitatively scored for the kidney lesion according to the literature. A total of 5 indexes were used to quantify renal toxicity: (a) cell infiltration; (b) degeneration of epithelium; (c) tubular necrosis; (d) edema; and (e) interstitial inflammation. The total score (TS) was calculated according to the equation: TS = a + b + c + d + e. The scoring scales were defined as follows: 0, no toxicity; 0.5–1.0, mild toxicity; 1.0–2.0, moderate toxicity; above 2.5, severe histologic toxicity.

Statistical Analysis Statistical analysis was carried out by means of LSD t-test on average±standard deviation with SPSS 11.5. p<0.05 is considered as significant and p<0.01 highly significant.

RESULTS

Contents of AAs in the Extracts Based on the modified method, the content of four main AAs in the aqueous extracts of AF and HAF were determined. As shown in Fig. 2, baseline separation was obtained for the target compounds. The contents of AAs were summarized in Table 2.

Acute Toxicity In preliminary test, the 0–100% mortality doses range were found with D_{min}=20.2 g/kg/d, D_{max}=84.0 g/kg/d for AF, and D_{min}=41.0 g/kg/d, D_{max}=100.0 g/kg/d for HAF. Then the normal doses (Table 1) were selected in the following experiment.

Within 30 min of dosing, the independent activities of each group mice were significantly reduced, the animals showed shortness of breath first and then breath tended to deepen and be gentle; the ptosis closing, occasional convulsion phenomenon and reversal reflection were also observed. Along with the continuous doses, the mice developed dim and rough hair.

Table 2. Contents of Four AAs in the Aqueous Extacts of AF and HAF by HPLC (n=3)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content^b (mg/g)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA C</td>
<td>AA D</td>
</tr>
<tr>
<td>AF</td>
<td>0.632±0.015</td>
<td>0.225±0.008</td>
</tr>
<tr>
<td>HAF</td>
<td>0.586±0.013</td>
<td>0.182±0.005</td>
</tr>
</tbody>
</table>

^a Converted to the content in raw herb. ^b AAs = AA C + AA D + AA II + AA I

Table 3. Mortality of Different Oral Doses of AF

<table>
<thead>
<tr>
<th>Group</th>
<th>Oral dose (g/kg/d) lg(d)</th>
<th>Death time (d)</th>
<th>Mortality (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>84.0 1.92</td>
<td>0 0 1 2 4 1 1</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>58.8 1.76</td>
<td>0 0 0 1 3 4 0</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>41.2 1.61</td>
<td>0 0 0 0 3 2 0</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>28.8 1.46</td>
<td>0 0 0 0 0 3 2</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>20.2 1.30</td>
<td>0 0 0 0 0 1 1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

^a lg(d): the logarithm of dose valve.

Table 4. Mortality of Different Oral Doses of HAF

<table>
<thead>
<tr>
<th>Group</th>
<th>Oral doses (g/kg/d) lg(d)</th>
<th>Death time (d)</th>
<th>Mortality (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100.0 2.00</td>
<td>0 0 4 3 3 0 1</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>80.0 1.90</td>
<td>0 0 2 3 1 1 0</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>64.0 1.81</td>
<td>0 0 0 1 2 2 0</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>51.2 1.71</td>
<td>0 0 0 1 1 1 0</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>41.0 1.61</td>
<td>0 0 0 0 0 1 0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

^a lg(d): the logarithm of dose valve.
Abdominal breathing, prone and twitching phenomenon were observed before the animal’s death.

The number of deaths in mice was recorded within 7d (see Tables 3, 4). LD₅₀ of AF and HAF were calculated with improved Kow’s method. LD₅₀ of AF is 34.1±7.2 g/kg/d (p=0.95), and LD₅₀ of HAF is 62.6±8.0 g/kg/d (p=0.95).

**Subacute Toxicity** During the whole stage of dosing, no significant toxic signs and symptoms were found between the HAF group and the CON group. After the 2nd week, some animals showed depression, reduced movement, dim and rough hair. The emergence and severity of the above symptoms were in the following order: AF-H, AF-M, HAF-H, AF-L and HAF-M group. During the doses, there was no significant difference between the dose groups and the CON group in body weight (p>0.05). It was observed that two high groups (AF-H, HAF-H) had more urine output than other groups did.

The data of serum assay is shown in Table 5. There was a dose-dependant increase in SCr and BUN level after drug treatment. A significant increase was found in SCr level in AF-M (p<0.05), HAF-H (p<0.05) and AF-H (p<0.01) groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>SCr (µmol/L)</th>
<th>BUN (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF-H</td>
<td>103.6±19.6**</td>
<td>13.02±0.97*</td>
</tr>
<tr>
<td>AF-M</td>
<td>80.28±23.7*</td>
<td>10.47±1.83</td>
</tr>
<tr>
<td>AF-L</td>
<td>73.12±15.11</td>
<td>9.81±0.62</td>
</tr>
<tr>
<td>HAF-H</td>
<td>74.36±19.02*#</td>
<td>10.38±1.75*#</td>
</tr>
<tr>
<td>HAF-M</td>
<td>66.93±20.67</td>
<td>9.93±1.69</td>
</tr>
<tr>
<td>HAF-L</td>
<td>60.12±16.57</td>
<td>9.40±0.35</td>
</tr>
<tr>
<td>CON</td>
<td>57.91±7.53</td>
<td>7.21±2.05</td>
</tr>
</tbody>
</table>

* p<0.05 and ** p<0.01 versus CON group. # (p<0.05) versus AF-H group.

![Fig. 3. Light Microscopic Images of Kidney Sections from Rats in 28 d Oral Dosing of AF and HAF (H&E, ×100)](image)

A: CON group; B: AF-L group; C: HAF-L group; D: AF-M group; E: HAF-M group; F: AF-H group; G: HAF-H group.
compared to the CON group. There was a significant increase of BUN level in AF-H group compared to the CON group. Compared with AF-H group, HAF-H group showed a significant decrease in SCR and BUN level. SCR and BUN levels are important indicators of renal function. As shown in Table 5, the nephrotoxicity of AF and HAF are positively related with the oral doses, and the renal lesion of the HAF rats was milder than that of AF group.

The ROW ratios of liver were not significantly different between the groups. The AF-H group showed decreases in the ROW of kidney ($p<0.05$), and no significant differences of the ROW of kidney were found between the other groups with the CON group.

Nephrotoxic effects of AF and HAF on the rats are illustrated in Fig. 3. The results show that the structure of the renal glomerulus and kidney tubules in CON group were normal (Fig. 3A). In AF-L group (Fig. 3B) diffuse degeneration of a few proximal tubular epitheliums (PTE) were observed with mild infiltration of lymphocytes. In HAF-L group (Fig. 3C), less degeneration of epithelium occurred than that in AF-L group. In AF-M group (Fig. 3D) the structure of the renal glomerulus showed slight damage, some cell were hardening, and the proliferation of the cells and large ovoid cells were observed; the tubules in renal papilla expanded with structure being damaged; the inflammatory cells infiltrated into the interstitium. In HAF-M group (Fig. 3E), the structure of the renal glomerulus and tubules were mainly normal; some PTE were cavitory degeneration and some were necrotic; the vascular wall in interstitium was thickened. In AF-H group (Fig. 3F), the interstitium was hydropic degeneration accompanied with severe infiltration of inflammatory cells; the vascular wall was thickened and its cavity was necked as well as hyperemic; proximal convoluted tubules were widely degenerative and had some necrosis where they had some scattered protein tube types; the collagen proliferation was observed in the interstitium in medulla. In HAF-H group (Fig. 3G), the renal glomerulus had partial necrosis, the interstitium around the vascular was hydropic degeneration with the moderate infiltration of lymphocytes and protein accumulate; the renal tubules had some cavitory degeneration.

As a whole, pathological analysis showed that the renal lesions were mainly reflected in the structure injury of renal tubular, hyperemia, vacuolar degeneration and necrosis in the PTE. With the increase of the dose, the renal tubule was filled with the protein, and lymph cell inroad, interstitial fibrosis and collagen hyperplasia began to appear, even glomerular necrosis was found in the worst case.

Total histological scores are illustrated in Fig. 4, and the scores of various dosing groups are in the following order from high to low: AF-H, HAF-H, AF-M, HAF-M, AF-L, HAF-L and CON group. The histological results and scores indicated that the renal lesions increased as the dose increased. The nephrotoxicity of HAF was lower compared to the corresponding AF.

**DISCUSSION**

Traditional Chinese Medicine (TCM) has been used for thousands of years in China, and the curative effect and drug safety have always been its focus. Some drugs are marked with “slightly toxic,” “toxic” and “extremely toxic” in books on TCM. To eliminate or reduce the toxicity, drastic actions and side effects of some drugs, a series of theory and technique have formed through the long practice. Drug processing and compatibility are the main toxicity-removing methods. Some herbs containing AAs such as A. manushuresis(21,23,26) A. fangchi(25,26) and A. Ridix(31) have been reported about the possibilities of reducing toxicity with drug processing(29,31) and compatibility(21,25) As an anti-tussive and expectorant drug, the curative effect of AF is beyond doubt. In long clinical practice, the raw drug of AF has been found with some malignant characteristics, and then honey-frying technology was applied to attenuate the side effects of AF. So, there was a saying in TCM, “honey-frying makes AF sweet, mitigatory and moistens the lung.” After honey-frying, the contents of AAs decreased significantly (Table 2). The acute and subacute toxicity of the aqueous extracts of AF and HAF were also compared. All the investigated toxic signs and symptoms indicated that the nephrotoxic effects of HAF were lower than those of AF. The results suggested that that HAF should be safer than AF in clinical use. The experiments also validated the scientific connotation of the honey-frying technology of AF. Here, we recommend that HAF should be adopted in clinical medicine instead of its crude drugs (AF) because of its increased safety.

The subacute results showed a dose-dependant relationship of the nephrotoxicity of AF and HAF (Fig. 5). But the toxicity growth rate of AF was bigger than that of HAF. Even when the contents of AAs were equivalent, the toxic effect of AF was more serious than that of HAF. The probable explanation may be attributed to the differences in pharmacokinetics or toxicokinetics of AF and HAF, and need further investigation. The clinical doses of AF and HAF are 3.0 g/d for the adults. The acute toxicity showed that the LD$_{50}$ of AF (HAF) was
about 227 (417) times the clinical doses, and the LD$_{50}$ of HAF was approximately 1.84 times that of AF. The low dose in subacute test is 0.238 g/kg/d, and equivalent to 14.3 g/d for an adult of 60 kg which is about 5 times of the clinical dose. In this work, only mild toxicity was observed with the low dose group. Even in the AF-H group, only moderate toxicity was observed (see Fig. 4). So in this sense, the current clinical dose may be a safe one during short-term ingesting AF or HAF under the guidance of TCM theory.

AA I and AA II are often taken as the most toxic components in Aristolochiae plants, and the contents of AA I and AA II in AF are relatively lower than those of in A. manshurienensis and A. fangchi. Decoction is a very common form of Chinese pharmaceutical preparation. The dissolution rate of AAs in water decoction decreases significantly, and the toxicity of TCM further attenuates. In this work the preparation process of the water extracts of AF and HAF is similar to the decoction with TCM water. The above descriptions can partly elucidate why few AAN cases occur due to ingesting AF and HAF despite the relatively high contents of AAs in them.

In conclusion, the toxicity of AF was moderate in the investigated doses. Honey-frying and decoction with water can decrease the contents of AAs, and attenuate the toxic effects of AF. But on the other hand, even taking the honey-frying and decoction with water into consideration, the content of AA I will still be well above the strict limit of 200 ppm formulated by U.K. in 2005. Due to the complexities and diversities of the herbs, sufficient attention should be still paid to the safety of AF and HAF, and more clinical cases and toxicological data are in need.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (No. 81060326, 81260605 and 81160509), Jiangxi Provincial Natural Science Foundation (No. 2010GZY0163) and Jiangxi Provincial Education Department (No. GJJ09277).

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