Kuntai capsule attenuates premature ovarian failure through the PI3K/AKT/mTOR pathway

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Kuntai Capsule Attenuates Premature Ovarian Failure

Through the PI3K/AKT/mTOR Pathway

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Abstract

Ethnopharmacological relevance
Kuntai capsule (KTC), a type of herb formulas, was first described in the book of Shang Han Za Bing Lun in the third century. KTC has been widely used for the clinical treatment of menopausal syndrome. Considering that premature ovarian failure is also known as premature menopause, this study was designed to investigate the effects and mechanisms of KTC on a mouse model of premature ovarian failure.

Materials and methods
Forty-five female C57BL/6 mice were chosen for this study. Fifteen of the mice were separated into the Control group. The remaining thirty were used to establish the premature ovarian failure model by injecting intraperitoneally with 75mg/kg cyclophosphamide and then by randomly dividing the mice into two groups. One group was considered the Model group, the other group treated with the Kuntai capsule intragastrically every day for one week called the KTC group. After treatment, mice were sacrificed for sampling. The ovaries morphology of mice was observed by hematoxylin and eosin (HE) staining, and all follicles were counted under microscope. Western blotting was used to detect the PI3K/AKT/mTOR pathway activation. Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E\textsubscript{2}) and anti-mullerian hormone (AMH) levels were measured by enzyme-linked immunosorbent assay (ELISA). The fertility was observed by giving treated mice 8 weeks for breeding.

Results
We found that primordial follicle counts were increased in the KTC group compared to the Model group. The phosphorylation of PI3K, AKT, mTOR, 4E-BP1 and S6K in
the KTC group significantly reduced compared to Model group. Serum FSH and LH levels in the KTC group were decreased compared to the Model group, while, serum E₂ and AMH levels in the KTC group were increased compared with the Model group. The litter size in the KTC group was improved compared to Model group.

Conclusions
The KTC showed protective potentials of ovarian reserve and fertility to attenuate premature ovarian failure, which was relatively associated with activation of the PI3K/AKT/mTOR signaling pathway.

Keywords
Kuntai capsule, premature ovarian failure, Cell signaling, Ovarian function, Fertility

Abbreviations
KTC, kuntai capsule; POF, premature ovarian failure; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; AMH, anti-mullerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E₂, estradiol; S6K, S6 kinase 1; 4E-BP1, 4E binding protein-1; SPF, Specific Pathogen Free; CY, cyclophosphamide; PFA, paraformaldehyde; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TCM, traditional Chinese medicine.

1. Introduction
The reproductive longevity of women and most female mammals depends on the size of the ovarian primordial follicle pool ultimately, which is generally fixed during embryonic development or at birth(Richards & Pangas, 2010a, 2010b). During initial recruitment of follicles, primordial follicles are recruited from the reservoir of dormant follicles pool, the process termed follicular activation. Once entering the growing pool, ovarian follicles mature experienced from primary, secondary and antral stages to become pre-ovulatory follicles containing mature oocytes(McGee & Hsueh, 2000). During the entire process, a massive proportion of follicles undergo apoptotic death(atresia) without selection for further growth(Adhikari & Liu, 2009; Eppig, 2001; Kim, 2012). When the primordial follicle pool is exhausted, ovarian failure or menopause occurs. Anti-Mullerian hormone(AMH) is one of the most important measures of ovarian reserve, which is produced by the granulosa cells of pre-antral and small antral follicles, correlates with primordial follicle numbers in histology(van Rooij, et al., 2002).

Premature ovarian failure(POF) refers to amenorrhea in women less than 40-year old, accompanied by elevated menopausal serum gonadotropins levels(follicle-stimulating hormone, FSH>40IU/L) and decreased estrogen(Conway, 2000). POF is an ovarian dysfunction featured by premature depletion of ovarian follicles in nearly 1% of women under the age of 40 and 0.1% under the age of 30(Coulam, Adamson, & Annegers, 1986), which may lead to female infertility. Luteinizing hormone(LH) is a glycoprotein gonadotropin secreted by pituitary cells, it works together with FSH to promote follicular maturation, secrete estrogen, ovulation,
and the production and maintenance of the corpus luteum (de Koning, Popp-Snijders, Schoemaker, & Lambalk, 2000).

Chemotherapy is widely used for cancer and immune diseases treatment now. In recent years, the incidence of cancer has been increasing among younger adults, and more young women are suffering from immune diseases, receive chemotherapy. Although survival can be prolonged by chemotherapy, the side effects occur (Faubion, Kuhle, Shuster, & Rocca, 2015), among which chemotherapy-induced POF has been one of the most common. Low estrogen levels and infertility are the two major threats to POF patients, especially when they were at the child-bearing age (Kort, Eisenberg, Millheiser, & Westphal, 2014).

Studies have shown that PI3K signaling plays a significant role in folliculogenesis processes, including follicle activation, development and survival (Reddy, et al., 2008; Sobinoff, Nixon, Roman, & McLaughlin, 2012; Zheng, Nagaraju, Liu, & Liu, 2012), and POF is related to the decline of estrogen level (Etgen & Acosta-Martinez, 2003). Estradiol (E2) binds to estrogen receptor α (ERα) and β (ERβ) to participate in female sexual behavior. ERα binding to the regulatory subunit p85a of PI3K in a ligand-dependent manner induces phosphatidylinositol 3-kinase (PI3K) activity, leading to the activation of protein kinase B (PKB/AKT). AKT is known to result in the activation of mammalian target of rapamycin complex 1 (mTORC1) through multiple mechanism (Adhikari, Risal, Liu, & Shen, 2013). mTORC1 regulates cell growth and proliferation positively by integrating sorts of signals (Laplante & Sabatini, 2012). Stimulation of mTORC1 leads to over-activation of the entire primordial follicles pool, which caused POF and infertility (Adhikari, et al., 2009; Z. Chen, et al., 2015). Activation of mTORC1 promotes the phosphorylation of eukaryotic initiation factor 4E binding protein-1 (4E-BP1) and S6 kinase 1 (S6K1), those two are the downstream targets of the signaling, stimulating ribosome biogenesis and protein synthesis (Guertin, et al., 2006; Shahbazian, et al., 2006).

Kuntai capsule (KTC) is a type of herb formulas, consists of six traditional Chinese herbs, including Radix Rehmanniae Preparata, Rhizoma Coptidis, Radix Paeoniae Alba, Donkey Hide Gelatin, Radix Scutellariae, Poria. KTC has been proven to improve the peri-menopausal symptoms in post-menopausal women (Sun, et al., 2018; Zhang, Wang, & Yu, 2013; Q. Zhou, et al., 2016). In clinical studies, KTC also significantly improved symptoms in patients suffering from POF (Ma XX, 2012), and a combination of KTC, estrogen and progestogen had a higher cure rate (CC, 2009; Xu, 2010). Considering that POF is also known as premature menopause, this study was designed to investigate the effects of KTC on a POF mouse model via the PI3K/Akt/mTOR signaling pathway.

2. Materials and Methods
2.1. Animals and treatment

Female C57BL/6 mice (n=45, 8 weeks old, SPF grade, weigh 18-20g) were purchased from Shanghai Xipuer-Bikai Lab Animal Co., Ltd. The license number for animal manufacture was SCXK (HU) 2013-0016. Mice were fed in the SPF grade house with constant temperature (25°C) and light cycles (12-hour light, 12-hour dark).
Mice were treated according to the guidelines of Nanjing University of Chinese Medicine Animal Research Committee. Nanjing University of Chinese Medicine Biosafety and Animal Research Committees approved all experimental protocols.

The KTC is a product of Guiyang Xintian Pharmaceutical Limited Company (lot number 180314, Guiyang, China). The contents of KTC were carefully authenticated and standardized by Guiyang Xintian Pharmaceutical Limited Company (Jie, 2017; Yanfei, 2009). The herb details of KTC are presented in Table. The KTC was diluted to final contents of 50mg/ml with distilled water, and stored at 4°C before use.

The mice were divided into the Control group (n=15), the Model group (n=15) and the KTC group (n=15). The Control group received saline only. The Model group and the KTC group were injected intraperitoneally with 75mg/kg cyclophosphamide (CY, Aladdin, 6055-19-2). The KTC group was administered intragastrically at a dosage of 0.5g/kg per day for one week.

All the experimental animals were weighed and anesthetized with intraperitoneal injection of 10% chloral hydrate (350mg/kg) (Aladdin). After collecting blood samples for hormonal assays, the mice were euthanized. The ovaries were removed and weighed, then half of ovaries was snap-frozen in the liquid nitrogen, and stored at -80°C for further analysis, another part was fixed in 4% PFA.

2.2. Histological morphology observation and follicle classification and calculation

The ovarian tissues were fixed in 4% PFA at 4°C overnight, subjected to conventional dehydration in ethanol and cleared in xylene. The processed tissues were finally embedded in paraffin for consecutive sectioning. Ovarian tissues were sliced in 6-µm and sections are taken from five intervals 100-µm apart throughout each ovary. At each interval, five sections were cut in series and the first slide was stained with HE. Follicle counts were performed at five 100-µm intervals, which were deemed equivalent to whole-ovary sections. The number of follicles at four stages was counted by two investigators. Primordial follicles were observed at 40× magnification, primary, secondary and antral follicles at 20×. For the purposes of analysis, “growing” follicles were considered of all primary and secondary follicles. Follicles were counted by the average number of follicles per section as well as the average number of follicles per ovarian surface area (mm²). The area of each ovarian section was calculated by multiplying two perpendicular diameters measurements (mm²).

2.3. Western blotting

Proteins from ovary tissues were separated in 4-20% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The PVDF membranes were blocked in a solution containing TBST (10mM Tris-HCl, Ph7.4, 150mM NaCl, and 0.05% Tween 20) and 5% nonfat dried milk at room temperature for 1h, and incubated with PI3K (CY5355, 1:1000 dilution; Abways), phospho-PI3K (CY6427, 1:1000 dilution; Abways), AKT (9272, 1:1000 dilution; Cell Signaling Technology, USA), S473 phospho-AKT (9271, 1:1000 dilution; Cell Signaling Technology, USA), mTOR (YT2913, 1:1000 dilution; ImmunoWay), phospho-mTOR (YP1220, 1:1000 dilution; ImmunoWay), S6 (2217, 1:1000 dilution; Cell Signaling Technology, USA),...
phospho-S6(2211, 1:1000 dilution; Cell Signaling Technology, USA), 4E-BP1(9452, 1:1000 dilution; Cell Signaling Technology, USA), S65 phospho-4E-BP1(9451, 1:1000 dilution; Cell Signaling Technology, USA) at 4°C overnight. After washing with TBST buffer, the membrane was incubated with HRP-conjugated anti-rabbit antibody (7074, 1:3000 dilution; Cell Signaling Technology, USA) at a room temperature for 1h. After washing with TBST buffer, signals were detected by using a chemiluminescence reagent (Millipore, USA), imaged with a gel imaging system (Tanon, China), and quantified using Tanon image. Data are presented as the fold-change compared to the Control group.

2.4. Serum hormone level

The levels of serum FSH, LH, E2 and AMH were measured by mouse-specific ELISA kits (Nanjing Jin Yibai Biological Technology Co., Ltd., Shanghai, China) according to the manufacturer’s instructions (Catalog Nos. JEB-12673, JEB-12597, JEB-10719, JEB-12991). The concentration was determined by the absorbance at 450nm. Every experiment was done three repeats.

2.5. Mating protocol

The mating trials initiating from four weeks after treatment, mice were housed with proven male breeders (C57BL/6, 3- to 5-month). Once confirming signs of pregnancy, female mice were removed from the breeding cage. Separated female mice were monitored every 24 to 36 h in case of delivery. Pups were counted and weighed at birth. Following birth, data record included number of pups, weight of pups, percent of living pups and days from male interaction to birth. Mice were given up to eight weeks for breeding and successful birth of a first litter.

2.6. Statistical analysis

All data were analyzed by GraphPad Prism version 7.0 and presented as mean ± SEM. Results considered statistically significant are based on a criterion of P<0.05. The data were analyzed by one-way ANOVA, Student’s t test, chi-square test or Fisher’s exact test.

3. Results

3.1. Kuntai capsule protects the primordial follicle pool in POF mice.

Mean change in mouse weight from baseline, mean ovarian surface area and ovarian weight were used as flags for systemic and ovarian toxicity. Mouse weight in per group was similar at baseline. Mice in the Control group weighed more at sacrifice than they did at baseline (P<0.05). Mice in the Model and KTC group gained weight from baseline to sacrifice (Fig.1B). There were no differences when comparing ovarian weight and ovarian surface area (mm²) at sacrifice (Fig.1C and D) between three groups.

Following the schema, the effects were assessed of KTC on follicle counts. Primordial follicles per mm² were reduced in the Model group compared with the Control group (P<0.05). Mice in the KTC group trended toward more primordial follicles compared with mice in the Model group (P<0.05, Fig.2B). Primary and secondary follicle counts were not statistically different in the Control group and the KTC group, despite a trend toward fewer primary follicles and secondary follicles in
the Model group (Fig. 2C and D). There were fewer antral follicles per mm² in the Model group compared with the Control group (P<0.05, Fig. 2E). Ovaries of the Model group revealed a ratio of growing to primordial follicles more than that in the Control group, ovaries of the KTC group showed a ratio of growing to primordial follicles fewer than that in the Model group (P<0.05, Fig. 2F), all of these supporting our finding that KTC maintains ovarian quiescence and preserves the primordial follicle pool.

3.2. Kuntai capsule down-regulates the phosphorylation of PI3K/AKT/mTOR pathway proteins in ovaries of POF mice.

POF mice were treated with one-week KTC treatment and whole ovaries were harvested. CY added PI3K/AKT/mTOR pathway activation moderately, mainly shown by phosphorylation of PI3K, S473 AKT, mTOR, Thr389 S6 kinase and S65 4E-BP1 to P-PI3K, P-AKT, P-mTOR, P-S6K and P-4E-BP1. KTC reduced phosphorylation of PI3K, AKT, mTOR, S6K and 4E-BP1 (Fig. 3A). Downstream targets of the pathway were assessed by immunoblot analysis of the ratio of phosphorylated to total protein. The ratio of p-PI3K to PI3K was increased in the Model group compared with the Control group, then decreased after KTC treatment (P<0.05, Fig. 3B). The ratios of p-AKT and p-mTOR were increased in Model compared with Control (P<0.005), while the ratios were decreased after KTC treatment (P<0.05, Fig. 3C and D), then decreased after treated with KTC (P<0.05). P-S6K and P-4E-BP1 in the Model group were increased compared with the Control group, and declined after KTC treatment (P<0.05, Fig. 3E and F).

3.3. Kuntai capsule can up-regulate serum AMH, E2 levels, and down-regulates serum FSH, LH levels in POF mice.

To investigate the impact of KTC, serum FSH, LH, E2, AMH levels were measured by ELISA in all groups. The Model mice had significantly higher level of serum FSH than the Control mice (P<0.005), then the level decreased after KTC treatment (P<0.05, Fig. 4A). Serum LH level was significantly increased in the Model group than in the Control group, and the level was decreased after KTC treatment (P<0.05, Fig. 4B). Serum E2 level in the Model group were significantly lower than it in the Control group, and the level rose after KTC treatment (P<0.05, Fig. 4C). Serum AMH level in the Model group were significantly lower than it in the Control group (P<0.005), and the level increased after KTC treatment (P<0.05, Fig. 4D). The result above indicates that KTC can up-regulate serum AMH level to preserve the ovarian reserve function.

3.4. Kuntai capsule preserves fertility in POF mice.

We investigated whether KTC also preserves fertility in POF mice. Studies have shown that mice are able to start normal mating behavior at the time of 14 days following CY treatment (Jarrell, Bodo, YoungLai, Barr, & O'Connell, 1991). To ensure more rigorous, mice were harem-bred with proven male breeders and given 8 weeks to breed at 4 weeks following the final treatment. The Model group had a reduction in litter size compared with the Control group, and the KTC group had more litter sizes compared with the Model group (P<0.05, Fig. 5A). There were no differences in the percentage of pups live-born, pup weight (Fig. 5A and B). The time from beginning breeding to first birth was similar between groups, while there was a increasing trend
toward time from male introduction to birth in the Model group, which did not reach significance (Fig.S1C).

4. Discussion

POF is an early ovarian dysfunction in the clinic. It is defined as the ovarian dysfunction with hypo-estrogen levels and elevated gonadotrophin before or at 40. The main symptoms are the presence of primary or secondary amenorrhea for at least 4 months, low serum estrogen and elevated serum gonadotropin concentrations (FSH>40UI/I), and above all, loss of fertility (Shelling, 2010). The etiology and pathogenesis of POF is not yet known, and it is believed that it is mainly related to the permanent damage of ovaries. Chemotherapy is considered as one of the leading causes of POF, especially the wide use of alkylating agents, such as CY, the core of breast cancer chemotherapy, with high clinical toxicity, and induces ovarian damage by activating PI3K/AKT/mTOR Pathway, causing primordial follicle activation and follicular burnout, leading to loss of ovarian reproductive and endocrine function (Kalich-Philosoph, et al., 2013). The follicular burnout is referred to the ovarian exposure to chemotherapy repeatedly, damage to growing follicles, resulting in dormant primordial follicles to activate and grow to replace damaged antral follicles (Meirow, Biederman, Anderson, & Wallace, 2010).

Although POF was not nominally recorded in TCM, its clinical manifestations can be classified into the category of amnesic amenorrhea, blood dryness and infertility (Kou, Ding, Chen, Liu, & Liu, 2016). The TCM etiology includes insufficient transformation of blood of the spleen and stomach, severe consumption of yin blood and exhaustion of blood source. Its pathogenesis is mainly kidney deficiency, and dysfunction, while kidney deficiency is the most fundamental. KTC, a TCM herbal formulation, was derived from the Chinese masterwork Shang Han Lun, written by Zhang Zhongjing. KTC contains six kinds of Chinese medicine ingredients such as Radix Rehmanniae Preparata, Rhizoma Coptidis, Radix Paeoniae Alba, Donkey Hide Gelatin, Radix Scutellariae, Poria, with the effects of nourishing Yin, regulating Yin and Yang, stabilizing mind, reducing pathogenic fire and eliminating worries. Recent clinical trials in China have manifested significant positive effects of KTC in alleviating menopausal symptoms in Chinese women (Li, et al., 2010; L. L. Zhou, et al., 2009). Reports have indicated some molecular mechanisms about KTC, they found that KTC could increase the peripheral serum mRNA and protein expression of ERα and ERβ in peri-menopausal patients (J. M. Chen, et al., 2015). Considering that POF is known as premature menopause, this study was designed to investigate the effects of KTC on a mouse model of POF via the PI3K/Akt/mTOR signaling pathway.

At present, studies have shown that mice were injected in intraperitoneal with 75mg/kg of CY, all follicles in the ovary were reduced, and primordial follicles decreased to half (Spitz, et al., 2001). Therefore, a single intraperitoneal injection of 75mg/kg CY was used to establish a mouse model for ovarian dysfunction. Parameters assessed in our study included all follicles counts, ovarian morphology, serum hormone, protein expression, fertility. In the Model group, we observed some
changes in follicles counts, hormone levels, proteins expressions and fertility. Such findings indicate that CY can increase the phosphorylation of PI3K, AKT, mTOR, S6K and 4E-BP1, regulate serum AMH, E2, FSH, LH levels, reduce the primordial follicles, and have an impact on fertility. Following KTC treatment, the PI3K/AKT/mTOR signaling pathway was activated(Fig.5B), including decreased phosphorylation of the pathway proteins, recovery of serum AMH, E2, FSH, LH levels, more litter sizes. The ovarian function was protected and the fertility was improved.

In conclusion, KTC promotes ovarian function recovery and protects fertility in POF, which is associated with the activation of PI3K/AKT/mTOR signaling pathway.

Author’s contributions
ZH(619144564@qq.com) performed the experiments, analyzed the data and wrote the first draft of the manuscript. LZG(amen0614@126.com) and ZD(dzhang@nju.edu.cn) designed the research, supported the funding and revised the manuscript. QFF(475554619@qq.com), LAL(351534928@qq.com), SQM(1146651412@qq.com) and WQS(534616014@qq.com) analyzed and interpreted the data. LQ(hongseshan@163.com) and LSF(lushengfeng@njucm.edu.cn) provided theoretical and technical guidance. All authors read and approved the final manuscript.

Figure Legends
Fig.1. (A)An experimental schema.(B)Mouse weight at baseline and sacrifice(g). All mice gained weight from baseline to sacrifice: the Control group(*P<0.05), the Model group (n.s., P>0.05), the KTC group(n.s., P>0.05). (C)Ovarian weight at time of sacrifice(mg)(n.s., P>0.05). (D)Ovarian surface area(mm²)(n.s., P>0.05).
Fig.2. The KTC protects the primordial follicle pool in ovaries of the Model group mice.(A)Ovarian sections stained with H&E and representative images are shown (20x, 40x magnification). Representative follicles are marked with yellow arrows.(B)Primordial follicles were reduced in the Model group mice compared with the Control group mice (*P<0.05). The KTC group mice trended toward more primordial follicles compared with the Model group mice(#P<0.05).(C, D and E)Primary follicle, secondary follicle and antral follicle counts were not statistically different in the Control group and KTC group despite trending toward fewer primary follicles, secondary follicles and antral follicles in the Model group(n.s., P>0.05).(F)Ovaries of the Model group revealed a ratio of growing to primordial follicles more than that in the Control group (*P<0.05), ovaries of the KTC group showed a ratio of growing to primordial follicles fewer than that in the Model group (#P<0.05).
Fig.3. The KTC down-regulates downstream targets of the PI3K/AKT/mTOR pathway in the ovaries.(A)The representative immunoblots. (B)The ratio of p-PI3K to PI3K was increased in the Model group compared with the Control group(*P<0.05) , then decreased after treated with KTC(#P<0.05).(C)The ratio of p-AKT to AKT was increased in the Model group compared with the Control group(**P<0.05) , then
decreased after treated with KTC(#P<0.05).(D)The ratio of p-mTOR to mTOR was increased in the Model group compared with the Control group(**P<0.05), then decreased after treated with KTC(#P<0.05).(E)Phosphorylation of S6K was increased in the Model group compared with the Control group(*P<0.05), while KTC decreased phosphorylated S6K levels without affecting total protein content(#P<0.05).(F)Phosphorylation of 4E-BP1 in the Model group was increased compared with the Control group(*P<0.05), and reduced after treated with KTC(#P<0.05). Results are quantified from three series of representative immunoblots.

Fig.4. Serum FSH, LH increase and serum AMH, E2 decrease in the Model group mice whereas the KTC maintain their concentration. (A)The Model group mice had significantly higher levels of serum FSH compared with the Control group(**P<0.005), while the level declined after treated with KTC(#P<0.05). (B)Compared with the Control group, CY slightly up-regulates the serum LH(*P<0.05), then decreased after treated with KTC(#P<0.05). (C)Serum E2 in the Model group had lower levels compared with the Control group(*P<0.05), however, the levels increased after treated with KTC(#P<0.05). (D)Serum AMH in the Model group had lower levels compared with the Control group(**P<0.005), however, the levels increased after treated with KTC(#P<0.05).

Fig.5. (A)The number of the first pups litters. CY reduces litter size compared with the Control group(*P<0.05), and the KTC group had more litter sizes than the Model group(# P>0.05). (B) The PI3K/AKT/mTOR signaling pathway and a diagram of the sequence of development of follicles. Physiologic ovarian folliculogenesis proceeds from the primordial follicle stage, where an oocyte is activated, grows and transitions to a primary follicle, a secondary follicle and a antral follicle. Primordial follicles are surrounded by a single layer of fusiform granulosa cells; primary follicles are characterized by at least three single layers of shape granulosa cells; secondary follicles have at least two layers of granulosa cells; antral follicles have at least two layers of granulosa cells and a follicular antrum.

Fig.S1. (A)The percentage of pups live-born(n.s., P>0.05). (B)Pup weight(n.s., P>0.05). (C)Time from male introduction to birth(days). The Model group mice trended toward a longer time to birth, but this did not reach significance (n.s., P>0.05).

References


Table 1. The Chinese herb drugs contained in Kuntai capsule.

<table>
<thead>
<tr>
<th>Chinese name</th>
<th>English name</th>
<th>Latin name</th>
<th>Family</th>
<th>Plant part</th>
<th>Processing</th>
<th>Voucher number</th>
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<td>Baishao</td>
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<td>Paeonia lactiflora Pall</td>
<td>Ranunculaceae</td>
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<td>Dried</td>
<td>YP022</td>
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<td>Ejiao</td>
<td>Equus asinus L</td>
<td>Asini Corii Colla</td>
<td>Equidae</td>
<td>Hide gelatin</td>
<td>Stewed</td>
<td>YP024</td>
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<tr>
<td>Fuling</td>
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<td>Poria cocos Wolf</td>
<td>Polyporaceae</td>
<td>Sclerotium</td>
<td>Dried</td>
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<td>Coptidis Rhizoma</td>
<td>Coptis chinensis Franch</td>
<td>Ranunculaceae</td>
<td>Rhizome</td>
<td>Dried</td>
<td>YP021</td>
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<td>Scutellaria baicalensis Georgi</td>
<td>Labiatae</td>
<td>Root</td>
<td>Dried</td>
<td>YC028</td>
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<td>Rehmanniae Radix Praeparata</td>
<td>Rehmannia glutinosa Libosch</td>
<td>Scrophulariaceae</td>
<td>Root</td>
<td>Steamed with yellow wine</td>
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