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Safety evaluation of *Artemisia argyi* tender leaves as a food ingredient

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ABSTRACT

Artemisia argyi H. Lév. & Vaniot, commonly known as Chinese mugwort, is a wild vegetable with a long history of dietary use in China, and Qiai is its cultivated variety. However, it has not yet been included in China food catalog. Based on traditional folk edible processing methods, we developed *A. argyi* tender leaf powder (AATLP) to evaluate its safety. Here, the food safety of processed AATLP was assessed through acute toxicity, genotoxicity, repeated dose 90-day oral toxicity, and teratogenicity tests. AATLP components and human consumption were analyzed and surveyed, respectively. Results showed that the oral half lethal dose of AATLP was greater than 7.5 g/kg body weight (BW), indicating that it is practically non-toxic. Genotoxicity results (Ames, micronucleus, chromosome aberration) were negative. The results of 90-day oral toxicity test showed the no-observed-adverse-effect level (NOAEL) of 8.0 g/kg BW, without toxicological changes. The teratogenicity test indicated a NOAEL of 6.45 g/kg BW. Human consumption survey revealed no adverse effects. In conclusion, the findings provide toxicological and human survey evidence to confirm that *A. argyi* tender leaves can be used in food preparation for human consumption.

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1. Introduction

Artemisia argyi H. Lév. & Vaniot, commonly known as "Chinese mugwort", has its tender buds and seedlings listed as edible wild vegetables in the *Flora of China*[1]. Since ancient times, Chinese people have developed a unique dietary culture of "fresh and tender leaves of *A. argyi* (FTLA) for food, dried and aged ones for medicine"[2]. This tradition was first documented in the *Shi Liao Ben Cao* (Materia Medica for Dietotherapy) from the Tang Dynasty[3]. To this day, FTLA is still used to prepare traditional foods such as Qingtuan and steamed bun in China, Japan and South Korea[4]. *A. argyi* is rich in various bioactive compounds. Its cultivated variety, Qiai, which is noted for superior quality in the *Compendium of Materia Medica*[5], contains significantly higher levels of volatile oils, flavonoids, and tannins than other varieties of *A. argyi*[6]. Modern pharmacology has revealed its multiple effects, including anti-tumor, anti-inflammatory, anti-oxidation, antibacterial, immune regulation, protection against cell damage, improvement of cognitive dysfunction, α-glucosidase inhibition, and improvement of obesity[7-8]. Widely distributed across China, which ranks as the world's largest cultivator of *A. argyi*, the planting area exceeded 40 000 hectares in 2021, with a market value of approximately CNY 39.5 billion[9]. Market enthusiasm in the food sector is evident. A local food product standard for Qingtuan, a popular seasonal *A. argyi*-based snack in Southeast part of China, was developed by Shanghai in 2012, which was followed by Anhui, Sichuan, and Jiangsu provinces to

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successively issue group standards in 2023 and 2024[10-13]. Our team also contributed to formulating a local food safety standard Hygienic Specification for the Processing of Edible Tender Leaves of *Artemisia argyi* (DBS42/021–2026) in Hubei Province, China, which was promulgated on February 2, 2026[14]. However, since its mature, dried and aged leaves were included as a herb medicine for external uses in the *Chinese Pharmacopoeia* in 1963[15], *A. argyi* has not been listed in food catalog. This absence severely restricts market access and the large-scale development of *A. argyi*-based foods.

The use of *A. argyi* as a food is controversial due to the uncertainty of its food safety. Ancient herbal classics predominantly regarded *A. argyi* as non-toxic. However, the *Chinese Pharmacopoeia* classifies it as "slightly toxic"[16]. Notably, the majority of toxicological investigations have concentrated on the medicinal, dried and mature leaves or their volatile oils[17-18], while overlooking the safety of the edible parts and their processing *via* traditional culinary methods. Studies indicate that the toxicity of *A. argyi* is primarily concentrated in its volatile oils, which exhibit hepatotoxicity, nephrotoxicity, reproductive toxicity, and neurotoxicity. The mechanism of hepatotoxicity primarily involves hepatotoxicity associated with the releases of hepatic enzymes in the circulation, induction of hepatocellular necrosis and edema, stimulation of oxidative stress[19], mitochondrial damages[20], metabolic interferences[21], and disruption of specific signaling pathways[22]. The volatile oil also increases the formations of micronuclei in the fetal liver and bone marrow micronuclei, and sperm abnormality in rodents[18]. Interestingly, not all volatile oils exhibit toxicity. Those extracted with petroleum ether are non-toxic[23], suggesting that toxicity may be attributed to specific constituents. For example, thujone is considered as a neurotoxin, causing convulsions or epilepsy *via* inhibiting the *γ*-aminobutyric acid A receptor[24-25]. Compounds such as camphor, caryophylline, and (−)-ningketone are also suspected of having potential toxicity[26-28]. However, no direct evidence confirms that these individual compounds are toxic within the context of *A. argyi* itself. Additionally, the safety evaluation of FTLA and its products after cooking remains virtually unexplored. This oversight mirrors a broader challenge in assessing many traditionally wild edible plants, where research often prioritizes medicinal uses over culinary ones. A case in point is *Artemisia capillaris*, another species in the Asteraceae family. While the *Flora of China* records its "tender twigs and leaves" as edible[1], the first and only safety study approached it from a medicinal perspective, demonstrates no significant toxicity after 13 weeks of subchronic and genotoxicity tests on its extract[29]. In contrast, *Eucommia ulmoides* leaves are much more fortunate. It has the same long-standing edible history and practice as *A. argyi*. Systematic toxicological evaluations (genotoxicity, acute toxicity, subacute toxicity, subchronic toxicity, long-term toxicity, etc.) have proven their safety, and they were officially included in the *Catalogue of Substances That Are Both Food and Traditional Chinese Medicinal Materials According to Tradition* in 2023, providing a reference for FTLA[30-31].

FTLA is typically picked around the Qingming Festival and consumed after boiling in alkaline water. Other wild plants can be treated with similar methods. For instance, *Toona sinensis* is best eaten in the form of tender shoots and needs to be blanched to reduce nitrite (NIT) and other harmful substances before consumption[32-33]. Thus, we speculate that the raw material selection and traditional cooking methods may be crucial for ensuring safe consumption. Previously, we optimized a traditional alkaline boiling process to prepare FTLA into materials for consumption. This procedure effectively reduced potentially harmful substances, and improved sensory qualities and nutrient retention. The results of acute toxicity tests using this material showed that the treated powder was practically non-toxic (median lethal dose (LD_{50}) > 7.5 g/kg body weight (BW)) in rats. It also positively regulated gut microbiota by promoting beneficial bacteria and inhibiting pathogens[34]. However, a comprehensive toxicological profile, including genotoxicity, subchronic toxicity, and teratogenicity, still needs to be established to fully support its safety as a food ingredient. Therefore, based on our prior findings, the current study aims to conduct a systematic safety assessment of alkaline-boiled *A. argyi* tender leaf powder (AATLP) through a series of toxicological tests, ingredient analyses, and human consumption survey, providing a solid basis for scientifically formulating safety standards and dose guidelines for the use of *A. argyi* in foods, thereby promoting the rational development and utilization of this valuable plant resource. This will fill the gap in safety data for the edible application of tender *A. argyi* leaves, clarify the range of safe edible doses and key control nodes, and directly provide scientific data supporting the inclusion of tender *A. argyi* leaves in the food catalog in China. Meanwhile, it will offer references for the formulation of standards and industrial development of *A. argyi*-based foods nationally and internationally.

2. Materials and methods

2.1 Plant material and chemicals

Batches of FTLA of Qiai, the cultivated variety of *A. argyi*, were harvested in April 2024 from the Huayuangang Planting base (35°0′34″ N 113°5′10″ E) in Qichun County (Hubei, China) and authenticated by Yang Wenguang from the Center for Plant Resource Survey, Evaluation, and Identification at the Kunming Institute of Botany, Chinese Academy of Sciences. The cleaned FTLA was mixed in a 10-fold mass of 1% $NaHCO_3$ solution, boiled for 2 min, then subsequently soaked in more than 10-fold volume of cold water for 30 min and dried at a low temperature (≤ 40 °C). Finally, the dried sample was pulverized into AATLP for experimental use. Additionally, a portion of the alkali-boiled FTLA, FTLA (without alkali treatment) and mature leaves (without alkali treatment) from the same batch were taken for compositional analysis.

Cyclophosphamide monohydrate (CPM) was purchased from Saen Chemical Technology Co., Ltd. (Shanghai, China). *Salmonella* Typhimurium strains (TA97a, TA98, TA100, TA102, TA1535) and S9 rat liver homogenate were obtained from Jiangsu Qishi Biotechnology Co., Ltd. (Jiangsu, China). Sodium azide was sourced from Amresco (Solon, OH, USA). 2-Aminofluorene and fenaminosulf were procured from Sigma-Aldrich (MO, USA). 1,8-Dihydroxyanthraquinone was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Mitomycin C (MMC) was bought from Meilun Biology (Dalian, China). Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 cell culture medium was obtained from Hyclone (Shanghai, China), and fetal bovine serum was purchased from Inner Mongolia Daxi Biotech Co., Ltd. (Inner Mongolia, China). Colchicine was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The reagent kits for alanine aminotransferase (ALT), blood urea nitrogen (BUN), triglycerides (TG), aspartate aminotransferase (AST), glucose (GLU), total protein (TP), creatinine (CRE), albumin (ALB), total cholesterol (TC), alkaline phosphatase

(ALP), gamma-glutamyl transferase (GGT) were purchased from Neusoft Witmann Biotechnology Co., Ltd. (Nanjing, China). Reagents for calcium ion (Ca^{2+}), sodium ion (Na^{+}), potassium ion (K^{+}), and chloride ion (Cl^{-}) were supplied by Beijing Bailongteng (Wuhan, China) Technology and Trade Co., Ltd. (Wuhan, China). Prothrombin time (PT) and activated partial thromboplastin time (APTT) reagents were provided by Shanghai Changdao Biotechnology Co., Ltd. (Shanghai, China). Urine test strips were purchased from Changchun Dirui Medical Technology Co., Ltd. (Changchun, China).

2.2 Animals experiment

All animal experiments were performed in compliance with the Animal Research: Reporting of *In Vivo* Experiments 2.0 guidelines and approved by the Institutional Animal Care and Use Committee of Hubei Provincial Center for Disease Control and Prevention, in accordance with EU Directive 2010/63/EU for animal experiments. SPF-grade Wistar rats (SYXK (E) 2022-0065) and KM mice (SCXK (E) 2020-0018) were obtained from the Hubei Experimental Animal Research Center (Wuhan, China) and housed under specific pathogen-free conditions in individually ventilated cages. Animals were maintained at 20−26 °C with a relative humidity of 30%−70% and 12/12-h light/dark cycle. Standard pellet feed (SCXK 2021-0011) and autoclaved water were provided *ad libitum*. In the repeated dose 90-day oral toxicity and teratogenicity tests, the AATLP was administered *via* diet at 2.5%, 5.0%, and 10.0% for the low-, middle-, and high-dose groups, respectively. No additional casein was added since AATLP contained 28.53% crude protein versus 18.92% in the basal diet. The experimental animals in other tests were fed the basal diet. The nutritional components in feed were detected according to experimental requirements. At the end of the experiment, the animals were euthanized via an intraperitoneal injection of sodium pentobarbital (200 mg/kg for rats and 150 mg/kg for mice).

2.3 Components analysis

The contents of total volatile oils, eucalyptol, borneol, and thujone in FTLA and mature leaves without being boiled in alkaline condition, FTLA being boiled in alkaline solution and soaked in cold water were determined and compared. Nutritional components, polysaccharides, and flavonoids in AATLP were also measured. Moisture was determined using Method 1 outlined in GB 5009.3−2016. Crude ash, crude protein, and crude fat contents were conducted employing GB 5009.4/5/6−2016, respectively. The carbohydrate was calculated as the remaining fraction after protein, fat, moisture, and ash contents were subtracted (100% − (protein + fat + moisture + ash))[35]. Crude fiber was determined according to GB 5009.10−2003. *β*-Carotene, vitamin C, and vitamin B_2 were quantified using Method 1 as described in GB 5009.83/86/85−2016, respectively. Amino acid content was determined according to the method prescribed in GB/T 18246−2019. Selenium, copper, aluminum, zinc, iron, calcium, and manganese were measured by Method 1 in GB 5009.93/13/182/14−2017, GB 5009.90/92−2016, and GB 5009.242−2017, respectively. Tin was determined according to GB 5009.16−2023, while K^{+} and Na^{+} were measured by GB 5009.91−2017. Crude polysaccharides were determined using the phenol-sulfuric acid spectrophotometric method as previously described[36]. Using rutin as the reference sample, the total flavonoid content was measured using spectrophotometry at a wavelength of 510 nm[37]. Total volatile oils were determined using Method 2204, and eucalyptol, borneol, and thujone were analyzed using the gas chromatography (Method 0521) according to the *Chinese Pharmacopoeia* (2020 Edition)[38].

2.4 Acute toxicity

The acute oral toxicity test was conducted using the "limit test" method outlined in the Chinese National Food Safety Standard (GB 15193.3−2014). A total of 20 SPF-grade Wistar rats, at 8 weeks old and weighing 180−220 g with half male and half female were selected. Animals were fasted for 16 h before the experiment, and then AATLP suspended in distilled water (the maximum concentration was 0.125 g/mL) was administered orally at a volume of 20 mL/kg BW within 24 h (gavage time points were 0, 4 and 8 h, respectively). The cumulative AATLP total dose of the 3 administrations was 7.5 g/kg of BW. Animals were observed for 14 days, recording signs of toxicity symptoms, mortality, and BW changes (weighing on days 1, 8, and 15). At the end of the experiment, surviving animals were euthanized, and a gross necropsy was performed to examine the body surface, thoracic and abdominal cavities, and cranial cavity organs for any abnormalities.

2.5 Genotoxicity test

2.5.1 Bacterial reverse mutation test (Ames test)

The mutagenicity of AATLP were evaluated on 5 *S.* Typhimurium strains (TA97a, TA98, TA100, TA102, TA1535) using the plate incorporation method from GB 15193.4−2014. A total of 5 dose groups were set up (initial test 128, 320, 800, 2 000, 5 000 μg/plate confirmatory test 8, 40, 200, 1 000, 5 000 μg/plate), along with the untreated control group (spontaneous reversion group), negative control group 1 (distilled water), negative control group 2 (dimethyl sulfoxide), and strain-specific positive controls. Without S9, TA97a/TA98/TA102 received fenaminosulf (50 μg/plate) TA100/TA1535 received sodium azide (2.0 μg/plate). With S9, TA97a/TA98/TA100 received 2-aminofluorene (10 μg/plate) TA102 received 1,8-dihydroxyanthraquinone (50 μg/plate) TA1535 received CPM (100 μg/plate). Each condition was tested in triplicate. Revertant colonies were recorded after 48 h of incubation under 37 °C.

2.5.2 Mammalian erythrocyte micronucleus test

Micronucleus test was performed according to GB 15193.5−2014. Based on the results of acute oral toxicity test, the LD_{50} of the test substance was > 7.5 g/kg BW, the maximum concentration that can be administered by gavage was 0.125 g/mL, and the volume was 20 mL/kg BW. The high, medium and low dosages of the micronucleus test were 2.500, 1.250 and 0.625 g/kg BW, respectively. A total of 50 SPF-grade KM mice (aged 4−5 weeks, weighing 25.0−35.0 g) were randomly divided into 5 groups (n = 10 per group, comprising 5 males and 5 females), including 3 dose groups (2.5, 1.25, 0.625 g/kg BW), a negative control (distilled water), and a positive control (40 mg/kg BW CPM). The test substance was administered orally twice within 30 h (24 h interval) at 20.0 mL/kg BW in mice. After the second administration

for 6 h, and femoral bone marrow smears were obtained, fixed, stained with Giemsa, and examined under oil immersion. For each animal, 2 000 polychromatic erythrocytes (PCEs) were scored for the micronucleus rate (‰). Additionally, 200 PCEs and normochromic erythrocytes (NCEs) were counted to determine the PCE/(PCE + NCE) ratio.

2.5.3 *In vitro mammalian chromosomal aberration test*

The mammalian chromosomal aberration test was conducted according to GB 15193.23−2014 using the Chinese hamster ovary cells from the China Center for Type Culture Collection (Wuhan, China). The test included 3 dose groups (2 500, 1 000, 400 μg/mL each with ±S9), a negative control group (distilled water, ±S9), and 2 positive control groups (15.0 μg/mL CPM, +S9; 0.8 μg/mL MMC, −S9). Cells were treated with Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 medium containing test compounds or controls for 4 h (±S9), then cultured in 10% fetal bovine serum at 37 °C with 5.0% CO_2 for 24 h. Before harvesting, colchicine (1 μg/mL) was added to incubate for 4 h. After that, the cells were harvested to prepare stained specimens. A total of 200 metaphase cells were analyzed for each dose group, and the types of chromosomal aberrations and the rate of chromosomal structural aberrations were counted (gaps and numerical aberrations were not included).

2.6 *Repeated dose 90-day oral toxicity test*

The repeated dose 90-day oral toxicity test of AATLP was performed according to GB 15193.13−2015. A total of 100 SPF Wistar rats (aged 4–5 weeks, weighing 60–80 g, 50 males and 50 females) were randomly allocated into 4 main groups (n = 10/sex/group) and 2 satellite groups (n = 5 per sex per group). The main groups consisted of the negative control group (main female control, MFC; main male control, MM-C), low-dose group (main female 2.0 g/kg BW, MFL; main male 2.0 g/kg BW, MML), medium-dose group (main female 4.0 g/kg BW, MFM; main male 4.0 g/kg BW, MMM), and high-dose group (main female 8.0 g/kg BW, MFH; main male 8.0 g/kg BW, MMH), while the satellite groups included the satellite negative control group (satellite female control, SFC; satellite male control, SMC) and satellite high-dose group (satellite female 8.0 g/kg BW, SFH; satellite male 8.0 g/kg BW, SMH). After 7 days of acclimation, all animals were treated for 90 days, with satellite groups observed for an additional 28 days post-dosing. The clinical symptoms were checked daily and ocular examinations were performed before and after the experiment. BW and food intake were recorded weekly, with food utilization rate calculated.

2.6.1 *Hematology, blood biochemistry, blood coagulation and routine urine examinations*

After overnight fasting, animals were anesthetized with intramuscular Zoletil®50 (10 mg/mL, 2.0 mL/kg). Blood was collected from the abdominal aorta into EDTA-K_2 (1 mL), sodium citrate anticoagulant (1.8 mL), and plain tubes (3−5 mL) for hematological, coagulation, and biochemical tests, respectively. A fully automated hematology analyzer (Sysmex XT-2000i) was employed to determine the absolute counts of red blood cells (RBC), platelets (PLT), and white blood cells (WBC), along with the percentage of WBC differential counts (basophils (BASO), neutrophils (NEUT), eosinophils (EO), monocytes (MO), and lymphocytes (LY). Additionally, hematocrit (HCT) and hemoglobin (HGB) levels were measured. PT and APTT were measured using the fully automated blood coagulation analyzer (Mindray C3510). Blood biochemical parameters including ALT, BUN, TG, AST, GLU, TP, CRE, ALB, TC, ALP, GGT, Ca^{2+}, Na^+, K^+, Cl^- were performed using a fully automated biochemical analyzer (Beckman AU680 model). Following visual inspection of the urine sample, a fully automated urine analyzer (Dirui H-800) was employed for urinalysis. The tested parameters included specific gravity (SG), acidity-alkalinity (pH), NIT, total urine protein (PRO), urine glucose (u-GLU), ketones (KET), leukocytes (LEU), urobilinogen (UBG), urine bilirubin (BIL), occult blood in urine (BLD).

2.6.2 *Histopathological examinations and organ weight measurements*

At the end of the experiment, all surviving animals were euthanized and necropsied for organ weight measurement and pathological examination. The organs weighed included the adrenals, ovaries, brain (cerebrum, cerebellum, brainstem), spleen, epididymides, testes, heart, thymus, kidneys, uterus, liver, and any masses. The organ/BW ratio (%) was calculated asfollows:

$$\text{Organ/BW ratio (\%)} = \frac{\text{Organ wet weight (g)}}{\text{Fasted BW (g)}} \times 100$$

For histopathology, in addition to the weighed organs, tissues are also taken from the lungs, stomach, mesenteric lymph nodes, pancreas, thyroid/parathyroid glands, pituitary, bladder, prostate, large intestine (cecum, colon, rectum), and small intestine (duodenum, jejunum, ileum). After 10% formalin fixation and paraffin embedding, the tissues were sectioned and stained with hematoxylin and eosin (H&E) for microscopical examination. Pathological lesions were typed and graded.

2.7 *Teratogenicity test*

The teratogenicity test was conducted according to GB 15193.14−2015. SPF Wistar rats (104 females, aged 9–10 weeks, 250–330 g; 52 males, aged 9–10 weeks, 360–500 g) were mated at a 2:1 female-to-male ratio. Pregnancy was confirmed the next morning by vaginal smear or plug, recording that day as gestation day (GD) 0. Due to the high crude fiber content of AATLP, which may raise the risk of gavage abortion and death, the test substance was mixed into the feed and single-cage rearing was adopted. The feed given to each rat and the remaining amount were precisely weighed every day. Pregnant rats were randomly divided into 3 dose groups (1.65, 3.35, 6.45 g/kg BW by actual intake) and a negative control group (basal diet). Positive control values came from the lab's historical database (Acceptance No.: ECDC (2023) 06002, conducted from September 2023 to November 2023). From GD 6–15, rats were feed the test substance or basal diet for 10 days. Clinical symptoms and poisoning status were recorded daily. BW was measured On GD 0, 6, 9, 12, 15, and 20. BW gain was calculated as terminal minus GD 6 weight. On day 20, pregnant rats were euthanized the gravid uterine

weight, corpora lutea, implantations, and live, dead, resorbed fetuses were recorded. Fetuses were examined for sex, weight, body length, malformations (external, skeletal, visceral) and malformed litters. Additionally, net maternal weight gain (necropsy weight − gravid uterine weight − GD 6 weight) and teratogenicity rate were calculated.

2.8 Human consumption survey

All experiments on human subjects were conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants voluntarily provided informed consent. Participation was not mandatory, and no personally identifiable information was collected. A cross-sectional survey (Application No. 2025031) was conducted in Qichun County (the primary planting area of Qiai) and the surrounding areas of Wuxue City and Xishui County in Hubei Province (China) to evaluate the safety of FTLA consumption in folk dietary practices. Our goal was to recruit local permanent residents aged 3–90 years, employing quota sampling to ensure representativeness in terms of age, gender, and urban/rural residence[39-40]. The quotas were determined based on data from the 2021 population estimates by the Huanggang Municipal Bureau of Statistics. This study developed a structured questionnaire titled "Questionnaire on Qiai Leaves Consumption History" comprising 30 items. Face and content validity of the structured questionnaire was evaluated by an expert panel ($n = 3$). Cronbach's α value of 0.81 showed good internal consistency of the questionnaire. Trained investigators conducted face-to-face interviews using structured questionnaires, supplemented by visual aids to enhance accuracy. The questionnaire covers population distribution characteristics, dietary behaviors (consumption frequency, daily intake, consumption duration, consumed plant parts, consumption method), and adverse reactions (free reporting combined with a checklist method, defined as symptoms reported within 24 h of consumption). Consumption frequency was classified as low frequency (≤ 1 time/month or occasionally every few months), medium frequency (2−3 times/month), high frequency (≥ 4 times/month), and festival/seasonal concentration (e.g., during Qingming Festival).

2.9 Statistical analysis

Data are presented as mean ± standard deviation (SD), managed using Microsoft Excel 2019 (Microsoft Co. Redmond, WA, USA), analyzed with SPSS 27.0 (IBM Co., Armonk, NY, USA), and visualized using GraphPad Prism 8.3.0 (GraphPad Software, San Diego, CA, USA). Continuous variables with normal distribution and homogeneity of variance were analyzed using *t*-test or One-way analysis of variance otherwise, Chi-square or non-parametric tests were applied. All analyses were sex-stratified using two-tailed tests ($\alpha = 0.05$).

3. Results

3.1 Animal feed components

With the increase in the proportion of AATLP added to the basal diet, the contents of crude protein, crude fat, and crude fiber showed an increasing trend, while the contents of moisture and crude ash decreased relatively (Table 1).

Table 1
Nutritional components (%) of the basal feed and the feeds for dose groups.

Component	Basal feed	2.5% AATLP	5% AATLP	10% AATLP
Moisture	8.20	8.11	8.02	7.84
Crude protein	18.92	19.16	19.40	19.88
Crude fat	4.30	4.46	4.63	4.96
Crude fiber	4.80	5.25	5.71	6.61
Crude ash	7.50	7.48	7.46	7.42

3.2 Nutrients and components analysis

The nutrition of AATLP is predominantly composed of carbohydrates (49.33 g/100 g) and crude protein (28.53 g/100 g), with a high fiber content of 22.92%. Additionally, AATLP is rich in 15 amino acids and 10 minerals, with calcium being the most abundant, followed by sodium and potassium. The contents of β-carotene and vitamin C are 23 792.56 μg/100 g and 7.95 mg/100 g, respectively. Furthermore, AATLP contains 4.67% crude polysaccharides and 4.13% total flavonoids (Table S1).

Fig. 1 shows the contents of total volatile oil, eucalyptol, borneol and thujone in FTLA and mature leaves of *A. argyi* with or without alkaline treatment. Without alkaline treatment, the total volatile oil in FTLA was 0.059 8%, merely 13.77% of that in mature leaves (0.434 3%). Eucalyptol and borneol in the FTLA were 0.007 9% and 0.004 3%, representing 23.87% and 27.92% of mature leaf levels (0.033 1% for eucalyptol and 0.015 4% for borneol), respectively. The thujone content in FTLA was 0.008 1%, equivalent to 21.26% of the thujone level (0.038 1%) found in mature leaves. In FTLA after alkaline treatment, the total volatile oil content decreased to 0.006 7%, with no detectable eucalyptol, borneol, or thujone (detection limit: 0.000 6, 0.000 2, 0.000 5 mg/mL, respectively). This indicates an 88.80% loss of total volatile oils in FTLA after the alkaline treatment, along with reductions rates exceeding 92.41%, 88.37%, and 97.53% for eucalyptol, borneol, and thujone, respectively.

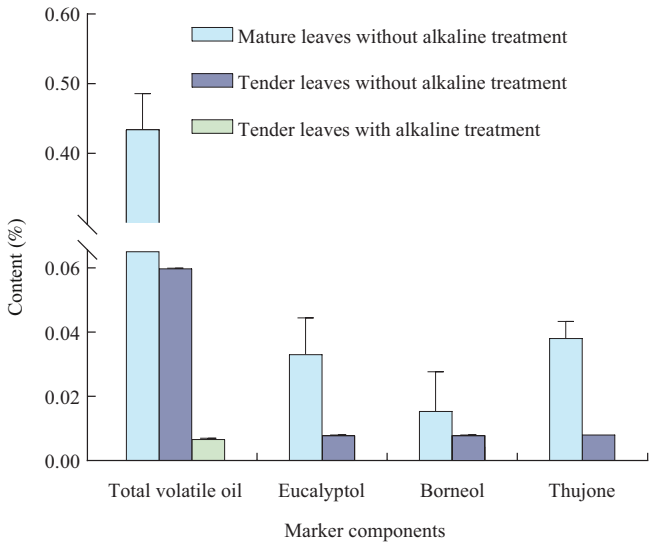


Fig. 1 Comparison of marker components in tender and mature *A. argyi* leaves with or without alkaline treatment. Eucalyptol, borneol and thujone were below the detection limit in the tender leaves after alkaline treatment. Values expressed as mean ± SD.

3.3 Acute toxicity

In this experiment, no poisoning symptoms were observed in the test animals. No statistically significant alterations in BW were observed after 14 days at a dose of 7.5 g/kg BW (Table 2). Gross necropsy demonstrated clean fur with no abnormal ocular, aural, oral or nasal secretions, and revealed no significant pathological changes in the major organs of thoracic and abdominal cavities or cranial structures. Therefore, the oral LD_{50} was > 7.5 g/kg BW, indicating a practically non-toxic classification.

Table 2

Results of acute toxicity in rats conducted with AATLP.

Sex	Initial BW (g)	BW at 1 week (g)	Final BW (g)	Deaths No.	LD_{50} (g/kg BW)
Female	183.0 ± 2.9	211.4 ± 5.7	227.9 ± 5.7	0	> 7.5
Male	184.7 ± 4.3	245.5 ± 11.0	291.6 ± 21.5	0	> 7.5

Note: values expressed as mean ± SD. n = 10 per group.

3.4 Genotoxicity test

3.4.1 Ames test results

Bacterial reverse mutation tests were carried out to assess the mutagenic potential of AATLP. In comparison to the spontaneous reversion and negative control groups, the quantity of *S.* Typhimurium (TA97a, TA98, TA100, TA102, TA1535, ±S9) at doses of 128−5 000 or 8−5 000 μg/plate did not show significant changes (P > 0.05) and did not surpass twice the count of spontaneous reversion colonies. These findings suggested a negative outcome for the mutagenesis test according to GB 15193.4−2014. Therefore, it can be concluded that AATLP does not induce genetic mutations at the tested concentrations (Table 3).

3.4.2 Mammalian erythrocyte micronucleus test

The micronucleus test was employed to assess whether AATLP induces chromosomal damage and to determine its mutagenicity. The

Table 3

Results of Ames test conducted with AATLP.

Dose (μg/plate)	Revertant colonies per plate									
	TA97a		TA98		TA100		TA102		TA1535	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Plate incorporation (initial assay)										
128	107 ± 6 (1.00)	109 ± 3 (1.11)	33 ± 6 (0.90)	32 ± 7 (1.14)	201 ± 29 (0.92)	220 ± 13 (1.06)	329 ± 31 (1.01)	338 ± 8 (1.03)	14 ± 2 (0.98)	16 ±1 (1.09)
320	111 ± 5 (1.04)	104 ± 5 (1.05)	34 ± 1 (0.94)	27 ± 1 (0.94)	223 ± 2 (1.02)	206 ± 14 (1.00)	325 ± 17 (1.00)	312 ± 7 (0.95)	15 ± 2 (1.07)	19 ± 4 (1.30)
800	106 ± 9 (0.99)	99 ± 5 (1.00)	37 ± 9 (1.01)	27 ± 1 (0.95)	196 ± 15 (0.90)	215 ± 7 (1.04)	326 ± 36 (1.00)	321 ± 17 (0.97)	14 ± 6 (0.98)	14 ± 78 (0.93)
2 000	116 ± 8 (1.08)	105 ± 5 (1.07)	34 ± 1 (0.93)	27 ± 3 (0.94)	244 ± 16 (1.12)	212 ± 16 (1.02)	317 ± 5 (0.97)	305 ± 37 (0.93)	14 ± 4 (0.98)	15 ± 3 (1.00)
5 000	112 ± 13 (1.05)	104 ± 5 (1.06)	40 ± 5 (1.10)	32 ± 3 (1.12)	198 ± 16 (0.91)	211 ± 8 (1.02)	307 ± 23 (0.94)	326 ± 38 (0.99)	18 ± 4 (1.26)	15 ± 8 (1.00)
Negative control 1	101 ± 4 (0.94)	102 ± 4 (1.03)	31 ± 5 (0.86)	31 ± 9 (1.09)	202 ± 11 (0.92)	218 ± 12 (1.05)	319 ± 31 (0.98)	321 ± 25 (0.97)	12 ± 1 (0.86)	17 ± 2 (1.18)
Negative control 2	104 ± 3 (0.97)	104 ± 7 (1.06)	33 ± 1 (0.91)	31 ± 4 (1.08)	186 ± 18 (0.85)	214 ± 15 (1.03)	304 ± 14 (0.93)	312 ± 18 (0.95)	11 ± 3 (0.79)	15 ± 3 (1.02)
Positive control	668 ± 158[a] (6.24)	677 ± 110[b] (6.86)	818 ± 51[a] (22.51)	909 ± 133[b] (32.07)	938 ± 96[c] (4.29)	887 ± 84[b] (4.28)	929 ± 97[a] (2.85)	877 ± 48[d] (2.66)	385 ± 80[c] (27.50)	449 ± 66[c] (30.61)
Spontaneous reversion	107 ± 6	99 ± 15	36 ± 3	28 ± 6	219 ± 14	207 ± 18	326 ± 10	330 ± 5	14 ± 3	15 ± 3
Plate incorporation (confirmatory assay)										
8	103 ± 8 (0.93)	106 ± 6 (1.02)	40 ± 6 (1.05)	37 ± 8 (0.81)	213 ± 16 (1.01)	228 ± 26 (1.02)	317 ± 14 (1.00)	303 ± 14 (0.97)	15 ± 4 (0.79)	16 ± 4 (0.84)
40	114 ± 2 (1.03)	112 ± 3 (1.08)	28 ± 2 (0.74)	38 ± 4 (0.83)	216 ± 6 (1.02)	220 ± 8 (0.99)	317 ± 10 (1.00)	291 ± 12 (0.93)	19 ± 5 (1.00)	16 ± 2 (0.86)
200	112 ± 6 (1.02)	98 ± 7 (0.95)	36 ± 6 (0.93)	41 ± 6 (0.89)	226 ± 36 (1.07)	222 ± 25 (0.99)	337 ± 20 (1.06)	315 ± 24 (1.01)	20 ± 3 (1.04)	18 ± 1 (0.95)
1 000	108 ± 12 (0.98)	108 ± 3 (1.05)	30 ± 2 (0.77)	37 ± 0 (0.81)	215 ± 3 (1.02)	228 ± 5 (1.02)	329 ± 9 (1.04)	293 ± 7 (0.94)	16 ± 2 (0.82)	16 ± 5 (0.86)
5 000	113 ± 10 (1.02)	107 ± 7 (1.04)	34 ± 2 (0.90)	35 ± 6 (0.77)	218 ± 14 (1.03)	222 ± 7 (0.99)	300 ± 19 (0.95)	303 ± 13 (0.97)	18 ± 3 (0.96)	18 ± 5 (0.98)
Negative control 1	113 ± 1 (1.02)	103 ± 3 (1.00)	29 ± 6 (0.77)	46 ± 5 (1.01)	209 ± 22 (0.99)	232 ± 6 (1.04)	315 ± 29 (0.99)	295 ± 24 (0.94)	14 ± 3 (0.75)	14 ± 2 (0.77)
Negative control 2	109 ± 6 (0.99)	104 ± 2 (1.00)	32 ± 3 (0.84)	36 ± 2 (0.80)	207 ± 19 (0.98)	216 ± 17 (0.96)	319 ± 10 (1.01)	326 ± 20 (1.04)	16 ± 2 (0.84)	17 ± 4 (0.89)
Positive control	746 ± 110[a] (6.76)	763 ± 98[b] (7.36)	775 ± 56[a] (20.23)	851 ± 97[b] (18.64)	925 ± 55[c] (4.38)	995 ± 184[b] (4.45)	997 ± 127[a] (3.15)	871 ± 38[d] (2.79)	417 ± 105[c] (21.95)	442 ± 36[c] (23.66)
Spontaneous reversion	110 ± 4	104 ± 12	38 ± 2	46 ± 2	211 ± 15	224 ± 14	316 ± 18	312 ± 20	19 ± 4	19 ± 1

Note: [a]Fenaminosulf, 50.0 μg/plate; [b]2-Aminofluorene, 10.0 μg/plate; [c]Sodium Azide, 2.0 μg/plate; [d]1,8-Dihydroxyanthraquinone, 50.0 μg/plate; [e]CPM, 100.0 μg/plate. The results are presented as the mean ± SD. The values in parentheses represent the fold increase in the mean number of colonies for the group compared to the mean number of colonies in the spontaneous reversion group.

proportion of PCEs to total erythrocytes in each dose group showed no significant difference compared with the negative control group, and was ≥ 20% of that in the negative control group (Fig. 2A, $P > 0.05$). Compared with the negative control group, the micronucleus rate of the positive control group was significantly increased ($P < 0.01$), while the micronucleus rates of bone marrow cells among different dose groups did not show significant changes (Fig. 2B). According to the National Food Safety Standard (GB 15193.5−2014), these results demonstrate that AATLP is negative in mammalian micronucleus tests.

3.4.2 Mammalian erythrocyte micronucleus test

The micronucleus test was employed to assess whether AATLP induces chromosomal damage and to determine its mutagenicity. The proportion of PCEs to total erythrocytes in each dose group showed no significant difference compared with the negative control group, and was ≥ 20% of that in the negative control group (Fig. 2A, $P > 0.05$). Compared with the negative control group, the micronucleus rate of the positive control group was significantly increased ($P < 0.01$), while the micronucleus rates of bone marrow cells among different dose groups did not show significant changes (Fig. 2B). According to the National Food Safety Standard (GB 15193.5−2014), these results demonstrate that AATLP is negative in mammalian micronucleus tests.

3.4.3 In vitro mammalian chromosomal aberration test

The *in vitro* chromosomal aberration test was employed to assess the potential of AATLP to induce mutations. Compared to the negative control group, the positive control group (±S9) exhibited a significant increase in the chromosomal aberration rate ($P < 0.01$). However, no significant changes ($P > 0.05$) were observed in any of the AATLP dose groups (±S9). These results demonstrate that AATLP is negative in mammalian chromosomal aberration test (Table 4).

3.5 Repeated-dose 90-day oral toxicity test

3.5.1 Clinical symptoms observation, ocular examinations, BW, food intake, and food utilization rate

No mortality or clinical symptoms of toxicity were observed in any group throughout the study. Before and after the experiment, ocular examinations revealed no abnormalities in all groups. The negative control group and the dose groups were not different in BW and food intake during the experiment (Figs. 3A-D, $P > 0.05$). However, the overall food utilization rate in the MFH group was significantly decreased from week 1 to 13 ($P < 0.01$), whereas that in the MMM and MMH groups demonstrated a significant decrease at week 5 compared with that in the negative control group (Figs. 3E-H, $P < 0.01$).

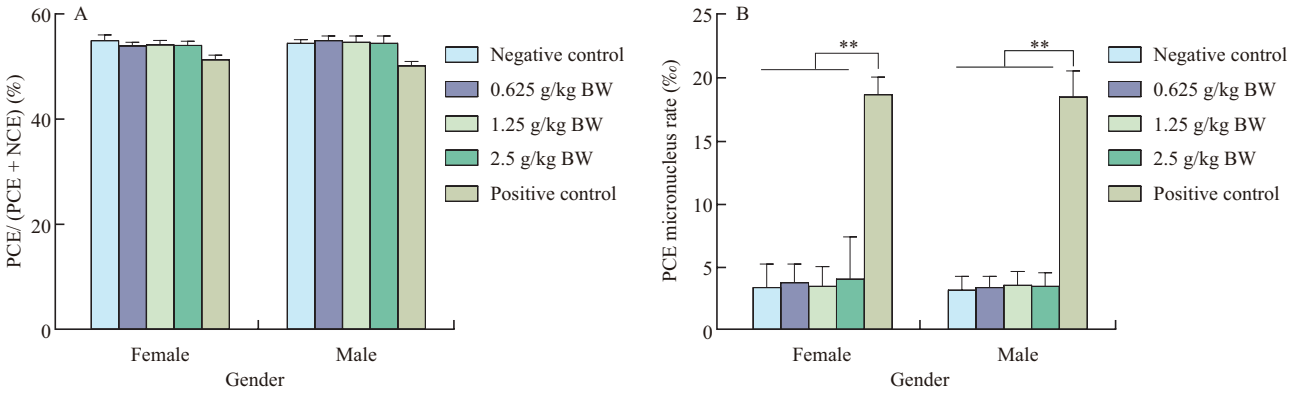


Fig. 2 Results of micronucleus test in KM mice. (A) Proportion of PCE among total erythrocytes; (B) Micronucleus rate in PCE. **Significantly different from the negative control at $P < 0.01$. $n = 5$ animals/group.

Table 4
Results of *in vitro* mammalian chromosomal aberration test with AATLP.

Groups	Dose (μg/mL)	Total cells scored No.		Aberrant cells No.[#]		Aberration rate[Δ] (%)	
		+S9	−S9	+S9	−S9	+S9	−S9
Negative control	0	200	200	7 (7,0)	6 (6,0)	3.5	3.0
Positive control	15.0 (CPM)	200	–	35 (18,2)	–	17.5**	–
	0.8 (MMC)	–	200	–	38 (22,0)	–	19.0**
AATLP	2 500	200	200	5 (5,0)	4 (4,0)	2.5	2.0
	1 000	200	200	4 (5,0)	3 (4,0)	2.0	1.5
	400	200	200	5 (3,0)	3 (4,0)	2.5	1.5

Note: –, Not tested or not applicable under the corresponding S9 condition; CPM was used only for +S9 and MMC only for −S9. ** Indicates a statistically significant difference compared with the negative control group ($P < 0.01$). [#]Number of cells with structural chromosomal aberrations (including counts of gaps, numerical aberrations such as polyploidy, and other aneuploid events). [Δ]The aberration rate calculation excludes gaps and numerical chromosomal aberrations. Aberration rate = Number of cells with structural chromosomal aberrations/200.

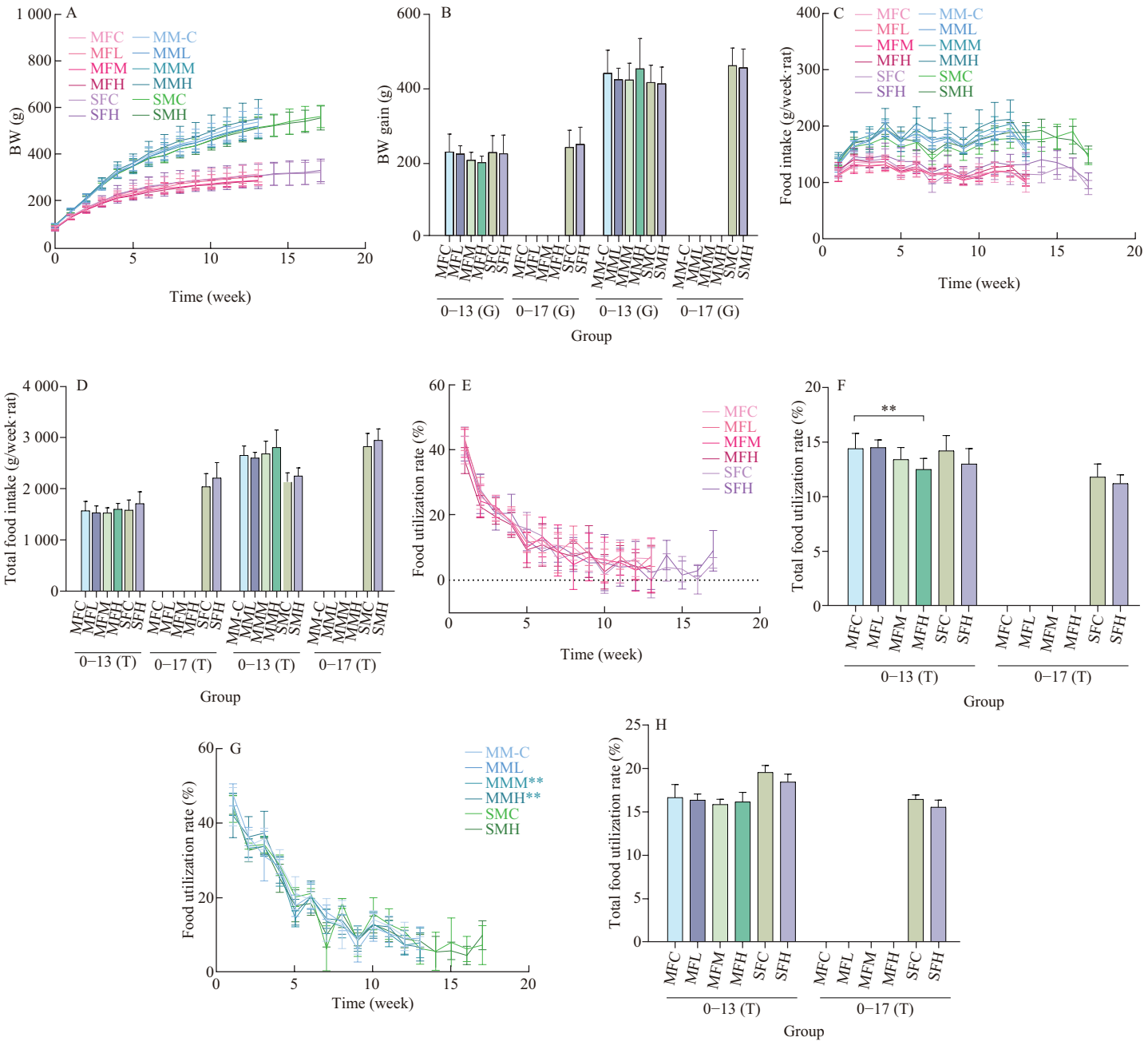


Fig. 3 Changes in BW, food intake, and food utilization rate of rats for 90-day repeated dose toxicity. (A-B) BW and BW gain of rats. 0–13 (G), the total weight gain from week 0 to week 13; 0–17 (G), the total weight gain from week 0 to week 17. (C-D) Food intake and total food intake of rats. 1–13 (T), total food intake from week 1 to week 13; 1–17 (T), total food intake from week 1 to week 17. (E-F) Food utilization rate of female rats. (G-H) Food utilization rate of male rats. 1–13 (T), total food utilization rate from week 1 to week 13; 1–17 (T), total food utilization rate from week 1 to week 17. ** Indicates significance at $P < 0.01$; specifically, in panel G, it denotes significant difference between the dose group and the negative control group at week 5.

3.5.2 Hematological and blood coagulation analysis

Compared with that in their respective control groups, LY ($P < 0.01$) and NEUT ($P < 0.05$) in the SFH and MMM groups were increased and descreased, respectively, whereas WBC counts were not fluctuated in all groups (Figs. 4A-C). The animals in SFH group (45-day) also exhibited elevated HGB ($P < 0.05$), RBC ($P < 0.05$), and HCT ($P < 0.01$) compared with that in the SFC group, while those parameters in the main groups (90-day) of both male and females were not changed (Figs. 4D-F, Tables S2-S4). No significant change was observed in satellite groups during the recovery period (Table S5).

3.5.3 Blood biochemical analysis and routine urine examinations

Transient fluctuations in proteins and electrolytes were observed in the 90-day dose group compared with the control group. In Figs. 4G-K, the SFH group (45-day) showed reduced ALB ($P < 0.05$), while the MFH group (90-day) exhibited decreased ALB ($P < 0.01$) and TP ($P < 0.01$). The MMM group (90-day) also demonstrated reduced ALB ($P < 0.05$). However, no changes were observed in hepatic injury markers (ALT, AST, ALP). Additionally, significant electrolyte elevation was observed in the 45-day satellite group (Figs. 4L-O).

The SFH group showed increased K^+ ($P < 0.05$), while the SMH group exhibited elevated Na^+ ($P < 0.01$) and K^+ ($P < 0.01$). No significant changes were detected in renal injury markers (Crea, BUN). During the recovery period, no group displayed significant alterations. Details and additional parameters are provided in Tables S6-S9. Tables S10-S13 indicate that there were no significant differences in urine parameters among all groups throughout the entire study ($P > 0.05$).

3.5.4 Necropsy, histopathological examinations and organ weight measurements

Both visual and microscopic examinations showed no abnormalities linked to dietary administration of AATLP. Tissues were predominantly normal in all groups, with only sporadic mild lesions observed in individual animals (Fig. S1). The occurrence of the lesions showed no significant difference between the test group

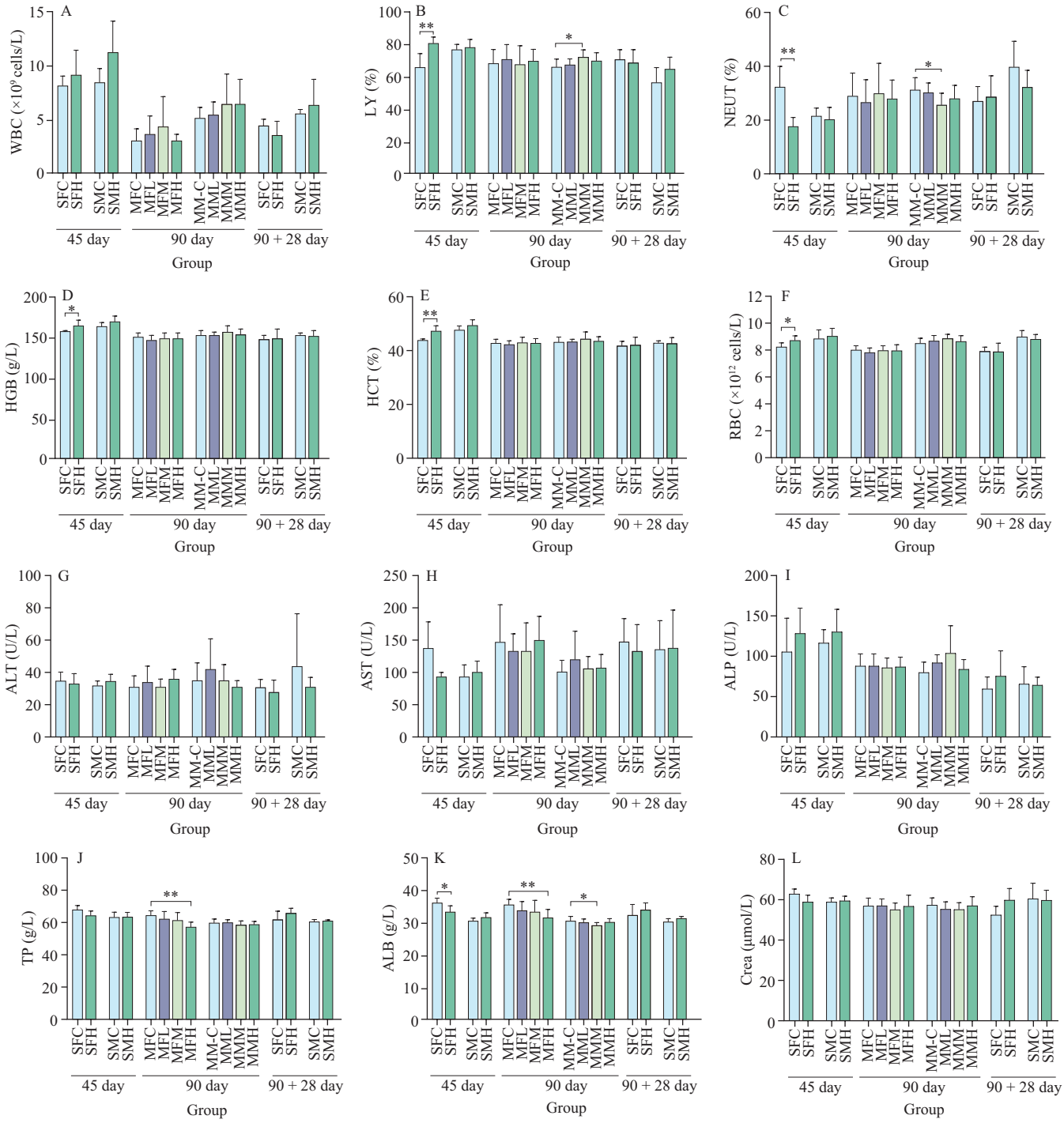


Fig. 4 Changes in hematology and blood biochemistry of rats in a 90-day repeated dose toxicity study. (A) WBC; (B) LY; (C) NEUT; (D) HGB; (E) HCT; (F) RBC; (G) ALT; (H) AST; (I) ALP; (J) TP; (K) ALB; (L) Crea; (M) BUN; (N) K^+; (O) Na^+. The results are presented as the mean ± SD. Statistical significance was indicated by $^*P < 0.05$, $^{**}P < 0.01$.

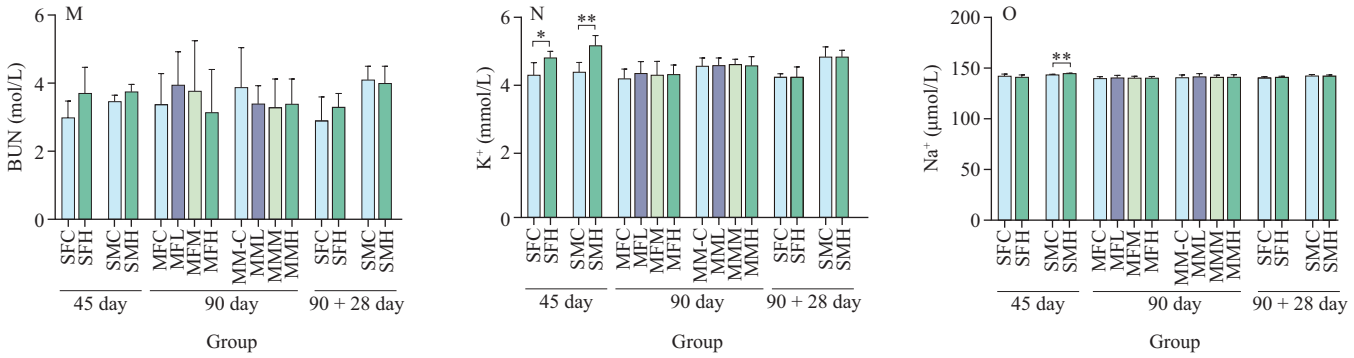


Fig. 4 (*Continued*)

and the negative control group, no gender-specific variation, and no significant improvement or deterioration during the recovery period (Table 5). Therefore, these lesions could not be attributed to the AATLP. The absolute and relative organ weights also showed no significant changes (Tables S14-S16). The no-observed-adverse-effect level (NOAEL) for both male and female rats was 8.0 g/kg BW.

3.6 Teratogenicity test results

The 10-day AATLP administration to pregnant rats did not induce any significant maternal toxicological symptoms. As shown in Tables S17-S18, no significant differences ($P > 0.05$) between any of dose groups within the range of 1.65−6.45 g/kg BW and the negative control group regarding the maternal BW, BW gain, net BW gain, corpus luteum count, implantations, live fetuses, resorbed fetuses, resorbed fetus rate, dead fetuses, and dead fetus rate was observed. Furthermore, no significant differences ($P > 0.05$) were detected among dose groups in gravid uterine weight, fetal body length, fetal weight, incidence of external malformations, litter incidence of external malformations, visceral malformation rate, litter incidence of visceral malformations, skeletal malformation rate, or litter incidence of skeletal malformations (Tables S19-S21). Therefore, AATLP exhibited no toxic teratogenic effects and the NOAEL was 6.45 g/kg BW.

Table 5
Summary of histological examination results for satellite group.

Stage	Sex	Lesion names	Satellite control group	Satellite 8.0 g/kg BW group	*P*
90 days	Female		*n* = 10	*n* = 10	
		Pulmonary foam cell aggregation	1	0	1.000
		Pulmonary ossification	0	1	1.000
		Hepatic inflammatory cell infiltration	1	0	1.000
		Mild hepatic steatosis	0	1	1.000
		Renal tubular hyaline casts	0	1	1.000
		Renal pelvis mucosal epithelial hyperplasia	0	1	1.000
		Uterine cavity dilation	1	1	1.000
		Increased thymic glandular structures	0	1	1.000
	Male		*n* = 10	*n* = 10	
		Pulmonary foam cell aggregation	0	1	1.000
		Pulmonary ossification	1	0	1.000
		Gastric gland dilatation	1	0	1.000
		Hepatic inflammatory cell infiltration	0	1	1.000
		Renal tubular hyaline casts	1	0	1.000
90 days + 28 days	Female		*n* = 5	*n* = 5	
		Myocardial inflammatory cell infiltration	0	1	1.000
		Hepatic inflammatory cell infiltration	1	0	1.000
		Renal tubular hyaline casts	1	0	1.000
		Renal tubular mineralization	0	1	1.000
		Adrenal spongiotic degeneration	1	0	1.000
		Residual thyroglossal duct remnant	1	0	1.000
		Increased thymic glandular structures	0	1	1.000
	Male		*n* = 5	*n* = 5	
		Myocardial inflammatory cell infiltration	2	0	0.444
		Gastric gland dilatation	1	0	1.000
		Hepatic inflammatory cell infiltration	1	0	1.000
		Mild hepatic steatosis	0	1	1.000
Total			14	12	

3.7 Human consumption survey

The survey collected 605 questionnaires with 600 valid responses (99.17% validity). Among respondents, 96.50% (579 individuals) consumed FTLA, primarily elderly over 60 (40.24%) and agricultural/manual workers (52.16%). Female consumers (54.92%) were slightly higher than that of males (45.08%). Most respondents were located in Qichun County (63.90%). Furthermore, the sample also included some maternal (13.30%) and chronic diseases patients (3.28%). *A. argyi* leaf was the primary part of the plant consumed (75.50%). The predominant consumption method includes pastries/wheat products (40.68%) and fresh consumption (18.57%) (Fig. 5A). Local residents typically harvest the FTLA from the top of the plant around the Qingming Festival, blanching them before using them in cold dishes, tea, or making Qingtuan. For gastrointestinal discomfort, fully grown and aged *A. argyi* leaves are often kneaded into medicinal pills for consumption.

Statistical analysis revealed significant differences in consumption frequency among different ages, jobs, and regions ($P < 0.001$). Obviously, children aged 3−12 years demonstrate a distinct propensity for festival/seasonal consumption, whereas adults prefer low to moderate-frequency consumption (Fig. 5B). In terms of consumption duration and daily, males exhibited longer consumption duration ($P = 0.000\ 4$), while females showed higher daily intake ($P = 0.017\ 9$). The consumption duration increased with age ($P < 0.001$), while the daily intake showed an inverted U-shaped distribution ($P = 0.000\ 6$), with the highest in young adults aged 18−35 years (10.62 g/day) and the lowest in children (7.85 g/day). Adults had an average consumption duration of 36.68 years and a daily intake of 8.2 g/day. Agricultural/manual laborers had the longest consumption duration ($P < 0.000\ 1$, 44.51 years), while "others" had the highest daily intake ($P < 0.000\ 1$, 11.03 g/day). Regional comparison showed that Xishui County was significantly prominent in both consumption amount ($P < 0.000\ 1$, 13.38 g/day) and daily intake ($P < 0.000\ 1$, 36.68 years). Among special populations, the maternal showed a relatively short average consumption duration (12.35 years), but their daily intake was relatively high at 11.38 g/day compared to other populations (Figs. 5C and D). This survey did not reveal any adverse reactions.

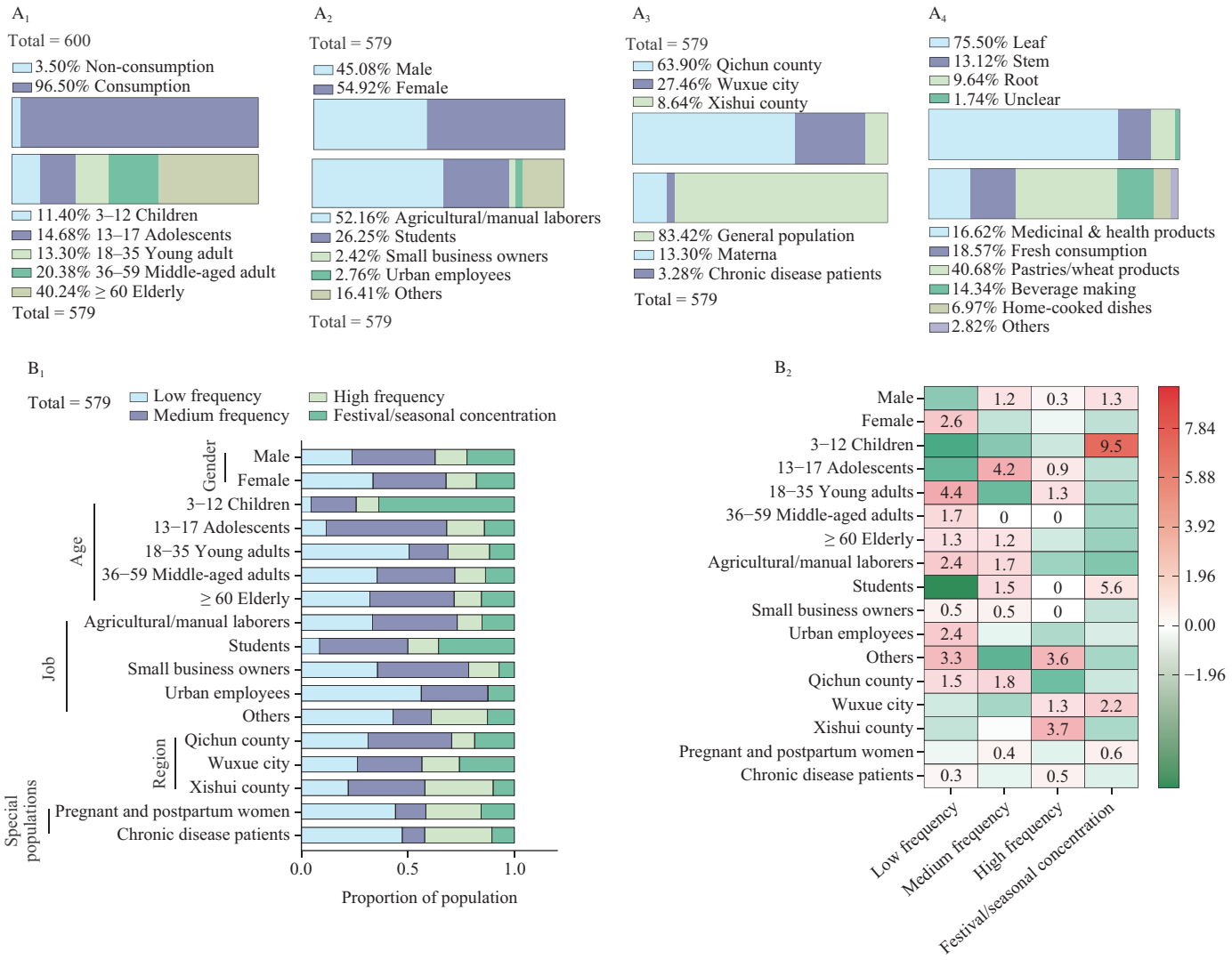


Fig. 5 Results of the human consumption survey on FTLA. (A) Population distribution characteristics; (B) Results of consumption frequency and residuals of different populations (Chi-square test standardized residuals indicate deviation from expected values); (C) Consumption duration of different populations; (D) Daily intake of different populations. The results are presented as percentages or mean ± SD. Statistical significance between groups was indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.000\ 1$.

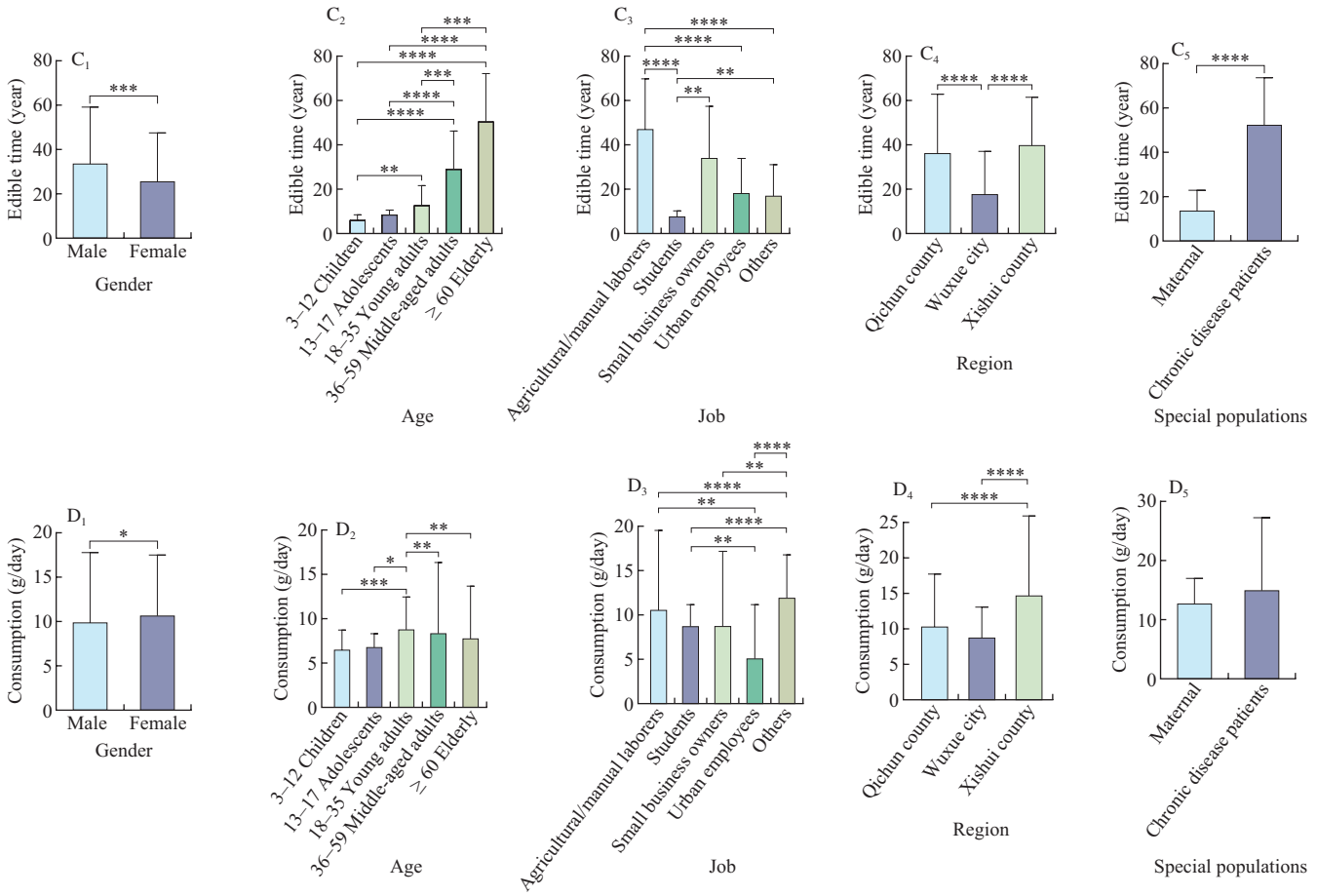


Fig. 5 (*Continued*)

4. Discussion

This study reveals and confirms the food safety of FTLA, which has been shown in its long history of dietary use. However, the *Chinese Pharmacopoeia* classifies mature and aged leaves of *A. argyi* as "slightly toxic", leading to persistent safety concerns. This can be attributed to the failure to differentiate between the raw material used for "culinary *A. argyi*" and "medicinal *A. argyi*," as well as research biases. The characteristics of FTLA significantly differ from that of the pharmacopeial mature leaves in terms of the edible parts, harvesting time, and processing methods. Culinary applications typically utilize the FTLA in the top 5−10 cm of the plant and harvested in the spring around the Qingmin Festival, then boiled in alkaline water before consumption. Whereas pharmacopeial applications of *A. argyi* often employ the fully grown and aged leaves harvested in the summer before flowering, dried and used as the traditional Chinese medicine. Component analysis confirmed these differences. The total volatile oil content in FTLA was only 13.77% of that in mature leaves. Notably, the content of the neurotoxic component thujone[40] in FTLA was merely 21.26% of mature leaves, while the contents of both eucalyptol (0.007 9%) and borneol (0.004 3%) were significantly lower than the minimally required amounts for medicinal use of *A. argyi* leaves as specified in the *Chinese Pharmacopoeia* (eucalyptol ≥ 0.05%, borneol ≥ 0.02%)[16]. Specially, the boiling in alkaline solution process is the key step in culinary attribution of *A. argyi* based food. Folk consumption practices reveal that this treatment may maintain the vibrant green color, soften the texture of the tender leaves, reduce bitterness and lower the concentration of volatile oils and certain pharmacological components[34]. The loss rates of total volatile oil, eucalyptol, borneol, and thujone in FTLA after alkaline boiling treatment are all over 88%, and the loss rate of thujone is greater than 97.53%. Consistent with our previous research[34], alkaline boiling effectively reduced the contents of eucalyptol, borneol, camphor, and α-thujone in FTLA to below the detection limits, providing a valid explanation for the absence of toxicity observed in this study. This may result from the breakdown of lignin and hemicellulose in cell walls, facilitating the release of chemical components[42-43]. Therefore, the FTLA naturally contains lower levels of potential risk substances, and therefore cannot be categorized as medicinal materials.

To assess the safety of the FTLA after alkaline boiling treatment, a systemic and comprehensive toxicological evaluation (acute toxicity, genotoxicity, 90-day subchronic toxicity, and teratogenicity) was conducted on AATLP. The results showed that all the test results were negative and no toxicological reactions related to the test substances were found. During the 90-day subchronic toxicity study, a transient reduction in food utilization was observed in the mid- and high-dose groups at certain time points, but there were no differences in food intake and BW. This can be attributed to the increased crude fiber content and other antinutritional factors introduced by AATLP in the diet, leading to a temporary decrease in digestive efficiency[44-45], which was considered a nutritional factor rather than a toxic effect.

Some mid- and high-dose groups showed a transient increase in LY and a decrease in NEUT, but WBC counts remained normal. Notably, the control group exhibited abnormally high NEUT and low LY, while the dose groups remained within normal ranges. This could be due to the stress-induced elevation of glucocorticoids in rats, leading to increased NEUT and decreased LY[46]. Therefore, the changes in the AATLP group's indicators were more likely to normalize the abnormally elevated NEUT and LY under stress, and may be related to the immune-modulatory activity of *A. argyi*[47]. Decreases in blood TP and ALB levels were also observed in some groups, but both were within the normal range of laboratory measurements (TP: 51.1−73.6 g/L; ALB: 25.7−44.0 g/L), There were no abnormalities in liver damage markers (ALT, AST, ALP)[48]. This phenomenon corresponds to the reduced feed utilization efficiency. It is hypothesized that the addition of AATLP increases the proportion of less digestibly plant-derived protein in the feed, thereby resulting in relatively low protein digestibility[49-50]. Additionally, the 45-day satellite group showed elevated serum Na^+ and K^+ levels in some high-dose groups, but there was no change in kidney function markers (BUN, Crea)[51], which is likely directly related to the presence of Na^+ and K^+ ions in AATLP itself. Furthermore, other individual hematological parameter changes were strictly within the normal reference range and did not show dose-dependency, thus were not considered to have toxicological significance or be related to AATLP exposure.

Results shown in this study confirm the safety and non-toxicity of AATLP. The fundamental reason for the reported toxic effect of fully grown and aged leaves of *A. argyi* for medicinal uses may be due to the differing nature of the research subjects. Previous references have primarily focused on the volatile oils extracted from *A. argyi* medicinal decoction pieces, reporting acute toxicity, genotoxicity, and significant hepatotoxicity. Liu et al.[18] found that the micronucleus rate in embryonic liver and bone marrow, as well as the sperm malformation rate, were significantly elevated in pregnant and male mice when administered *A. argyi* volatile oil at doses of 1 and 2 mL/kg *via* gavage, respectively, showing a dose-response. Furthermore, other studies have indicated that the toxicity of *A. argyi* volatile oil highly depends on the extraction method (with steam distillation being the most toxic), and the mechanisms of liver toxicity involve hepatocyte damage, mitochondrial damage, elevated serum ALT/AST/ALP levels, oxidative stress, metabolic disturbances, as well as disruption of the aryl hydrocarbon receptor and retinoid X receptor signaling pathways[19-23]. However, these studies typically involve complex mixtures, specific doses, and different preparation methods, making it difficult to directly extrapolate their conclusions to the consumption of edible FTLA processed through safe methods (boiling in alkaline solution). These reports serve as a reminder to pay attention to the risks in specific scenarios, but do not negate the safety of *A. argyi* consumption when using the correct raw materials and processing techniques.

As the main planting area of Qiai, Qichun County and its surrounding regions boast a long-standing tradition of folk consumption[52]. This study provides population-based evidence of the safety of traditional *A. argyi* consumption of FTLA through a systematic survey of 579 *A. argyi* consumers. The survey revealed that the average consumption duration of 579 consumers was 28.98 years (maximum 81 years), with an average daily consumption of 9.34 g (maximum 64 g). Among them, adults (≥ 18 years old) had a consumption duration of 36.68 years and a consumption amount of 8.20 g/day, and no adverse effects were observed regardless of the consumption part or method. Notably, although the consumption duration of pregnant and postpartum women was shorter (12.35 years), their daily consumption was higher (11.38 g). This may stem from local customs of using Qiai for perinatal care based on its traditionally recognized effects of "warming the meridians to stop bleeding and stabilizing the fetus"[16]. While these observational data cannot establish causal relationships or replace controlled toxicological studies, they provide valuable real-world context and population-based support for our experimental conclusions. The absence of reported adverse effects over hundreds of years of consumption, at intake levels relevant to human dietary practices, is consistent with and reinforces the safety profile established in our acute, genotoxicity, subchronic, and teratogenicity tests. This convergence of evidence from controlled laboratory models and long-term human consumption survey strengthens the overall conclusion regarding the safety of the traditionally processed FTLA.

5. Conclusion

This study integrates raw material identification, process analysis, component tracking, systematic toxicological evaluation, and investigation of human consumption to elucidate, for the first time, that differentiating raw materials (edible fresh and tender leaves versus medicinal mature dried leaves) and implementing key processing steps (alkali boiling) are central to understanding and ensuring the safety of *A. argyi* consumption. It demonstrates that FTLA boiled in alkaline solution poses no observable toxicological risk, and no adverse reaction was observed among ordinary consumers. It provides a solid scientific foundation for the safe utilization of *A. argyi* resources in both traditional and future novel food development. Next, we will work with other provinces across the country to formulate the industry standards for the FTLA consumption, providing a legal and safe access path for FTLA as a food ingredient and guiding the entire industrial chain to achieve standardized and high-quality development. Moreover, this study can provide safety evaluation materials for the inclusion of FTLA in the Chinese food catalogue. It is necessary to deeply explore the core health benefits of FTLA and its active components *in vitro* and *in vivo*, such as warming the meridians and stopping bleeding, intestinal health, and anti-aging effects. Through an active-oriented component screening platform, the basis of core active constituents can be analyzed, and molecular biology and omics methods can be used to clarify their action targets and mechanisms. Furthermore, in order to understand its long-term health effects among a broader population, it is necessary to broaden the survey of *A. argyi* use as food with that of other regions in China. Such research would facilitate the internationalization of *A. argyi*-based foods and the establishment of corresponding safety standards.

Conflict of interest

Guoxun Chen is an associate editor, and Fang Yang is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors declare no competing financial or non-financial interests related to this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2026.9251019.

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