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Acute and Sub-acute Oral Toxicity of *Trachyspermum ammi* Hydroalcoholic Seed Extract in Wistar Rats

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ABSTRACT

Background: *Trachyspermum ammi* (Ajwain) is widely used in traditional medicine across South Asia and the Middle East; however, systematic toxicological evaluations remain limited. This study assessed the acute and sub-acute oral toxicity of *T. ammi* hydroalcoholic seed extract (TAHE) in Wistar rats, with emphasis on hepatic safety.

Materials and Methods: Acute toxicity was evaluated according to Organisation for Economic Co-operation and Development (OECD) guideline 423 following a single oral dose of 2000 mg/kg in female rats (n=5). Subacute toxicity was assessed through repeated oral administration for 28 days at doses of 75, 150, and 300 mg/kg/day in male rats (n=10/group). Assessments included clinical observations, body weight changes, hematological and serum biochemical analyses, and histopathological examination of liver tissue.

Results: No mortality or treatment-related clinical signs occurred in either study. Body weight gain, food consumption, and hematological parameters remained comparable among the groups. Among the hepatic biomarkers, aspartate aminotransferase (AST) showed a statistically significant difference among groups (P=0.041); however, this difference was not maintained in post hoc pairwise comparisons with the control group (all P>0.55). Alanine aminotransferase (ALT), total bilirubin, albumin, and total protein remained unchanged. A dose-dependent increase in serum uric acid reached statistical significance at 300 mg/kg (P=0.009). Histopathological examination revealed preserved hepatic architecture, with no significant differences in congestion, hemorrhage, inflammatory infiltration, or degeneration scores (all P>0.35).

Conclusion: TAHE demonstrated a favorable safety profile, with an oral LD_{50} exceeding 2000 mg/kg and a no-observed-adverse-effect level (NOAEL) of 150 mg/kg/day in the 28-day study. The isolated elevation in uric acid at the highest dose warrants monitoring in longer-duration studies.

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Introduction

The liver is the principal organ responsible for xenobiotic metabolism, detoxification, bile secretion, and plasma protein synthesis [1]. Liver diseases cause approximately 2 million deaths annually worldwide, representing a substantial global health burden [2]. Conventional therapies, such as corticosteroids and antiviral agents often demonstrate limited efficacy and considerable adverse effects, motivating the search for safer and more affordable alternatives from medicinal plants [3].

Drug-induced liver injury (DILI) remains a major concern in both pharmaceutical development and clinical practice. Hepatotoxicity can occur through direct intrinsic toxicity, the metabolic bioactivation of xenobiotics that generates reactive intermediates, or immune-mediated mechanisms [4]. Serum aminotransferases—alanine aminotransferase (ALT) and aspartate aminotransferase (AST)—are the most sensitive and widely accepted biomarkers of hepatocellular injury, while bilirubin, albumin, and alkaline phosphatase provide complementary assessments of hepatic function [5, 6].

Medicinal plants have been utilized for millennia, and growing global interest in herbal medicines is driven by their cultural acceptability, accessibility, and perceived safety [7]. However, the assumption that natural products are inherently safe is unfounded. Documented cases of severe hepatotoxicity from herbal preparations—including pyrrolizidine alkaloid-containing plants, germander (*Teucrium chamaedrys*), and kava (*Piper methysticum*)—underscore the critical need for rigorous preclinical safety assessment before any botanical preparation can be recommended for therapeutic use [8, 9]. International regulatory guidelines, particularly those established by the Organisation for Economic Co-operation and Development (OECD), provide standardized frameworks for such toxicological evaluations [10].

Trachyspermum ammi (L.) Sprague (syn. *T. copticum*, *Carum copticum*), commonly known as Ajwain, Carom, or Ethiopian Cumin, is an annual aromatic herb of the Apiaceae family [11]. The plant grows to 60–90 cm in height and bears finely divided leaves and compound umbels of white flowers that produce small, ovoid, yellowish-brown fruits with a distinctive thymol-like odor [12]. Native to Egypt and the Eastern Mediterranean, it is now widely cultivated in Iran, India, Pakistan, and Afghanistan [13]. In Iranian Traditional Medicine, the seeds (designated as "Nanakhah") are extensively used as carminative, antispasmodic, diuretic, analgesic, antiasthmatic, and anthelmintic agents [14].

The pharmacological properties of *T. ammi* are attributed to its rich phytochemical composition. The essential oil, with a yield of 2–6%, is dominated by thymol (39–63%), accompanied by p-cymene (15–28%), γ-terpinene (13–24%), and smaller quantities of carvacrol, β-pinene, and limonene [15, 16]. The non-volatile fraction contains carbohydrates, proteins, lipids, fiber, and minerals, including calcium, phosphorus, iron, and manganese [17]. Preclinical studies have documented antimicrobial, antifungal, antiviral, anti-inflammatory, antihypertensive, gastroprotective, and hypolipidemic activities of *T. ammi* extracts and essential oil [18].

Despite its extensive traditional use and documented pharmacological activities, a comprehensive toxicological evaluation of *T. ammi* seed extracts—integrating hematological, biochemical, and histopathological assessments across multiple dose levels in both sexes as recommended by OECD guidelines—remains absent from the literature. The present study was therefore designed to systematically investigate the acute and sub-acute oral toxicity of *T. ammi* hydroalcoholic seed extract (TAHE) in Wistar rats, with particular emphasis on hepatic safety biomarkers and histopathology, and to establish a no-observed-adverse-effect level (NOAEL) for future pharmacological investigations.

Materials and Methods

Plant material and extract preparation

Mature dried seeds of *T. ammi* were procured from a reputable herbal market in Birjand, South Khorasan Province, Iran. Botanical authentication was performed at the Herbarium of the Faculty of Agriculture, University of Birjand (voucher specimen No. 248). Seeds were shade-dried at ambient temperature (22–25 ˚C), pulverized into a fine powder, and extracted by cold maceration in an ethanol-water mixture (70:30 v/v) for 72 h with continuous magnetic stirring. The filtrate was concentrated under reduced pressure using a rotary evaporator (Heidolph, Germany) at 40 ˚C and lyophilized (Dena Vacuum Industry, Model FD-5005-BT, Iran). The dry powder extract (TAHE) was stored at −20 ˚C until use. The final extraction yield was approximately 15% (w/w) relative to dry seed powder. The extraction protocol followed general principles for the phytochemical characterization of medicinal plants [19].

Experimental animals

Adult male and female Wistar rats (12 weeks old, 200–250 g) were housed in standard polypropylene cages under controlled conditions (temperature: 22±2 ˚C, humidity: 50–60%; 12-h light/dark cycle) with ad libitum access to standard rodent chow (Behparvar Co., Tehran, Iran) and water. A 7-day acclimatization period preceded all experiments. The study protocol was approved by the Institutional Animal Ethics Committee of Birjand University of Medical Sciences, and all procedures complied with institutional guidelines for the ethical care and use of laboratory animals [20].

Acute oral toxicity study (OECD 423)

The acute toxicity study was conducted according to OECD guideline 423 (acute toxic class method) [21]. Following an 8-h fast, a single oral dose of TAHE (2000 mg/kg body weight) was administered by gavage to one female rat. After 48 h of uneventful observation, the same dose was sequentially administered to four additional female rats (total n=5). A control group (n=5 females) received an equivalent volumes of normal saline (0.9% NaCl). Animals were monitored continuously for the first 4 h and twice daily thereafter for 14 days. Observations included general appearance, motor coordination, reflexes, signs involving the central and autonomic nervous systems, and body weight, which was recorded on days 0, 7, and 14.

Sub-acute oral toxicity study (28-day repeated dose)

Forty male rats were randomized into four groups (n=10/group) following OECD guideline 407 [20]: Group I (control) received normal saline, whereas groups II, III, and IV received TAHE at 75, 150, and 300 mg/kg/day, respectively, by daily oral gavage for 28 consecutive days. Dose selection was based on the following rationale. First, published acute toxicity studies on *T. ammi* extract have reported an oral LD_{50} exceeding 2000 mg/kg in rodents, classifying it as a low-toxicity substance under globally harmonized system of classification and labelling of chemicals (GHS) category 5. The highest dose of 300 mg/kg represents approximately 1/7 to 1/10 of the reported LD_{50}, consistent with OECD 407 recommendations that the highest dose should not cause mortality or severe suffering. Second, ethnopharmacological literature reports the traditional human consumption of *T. ammi* seeds in the range of 1–5 g/day (~14–70 mg/kg for a 70-kg adult). Using the FDA-recommended body surface area conversion factor (rat Km = 6, human Km = 37), the rat dose range of 75–300 mg/kg/day translates to a human equivalent dose (HED) of approximately 12–48 mg/kg/day, encompassing the traditional intake range with an appropriate safety margin. Third, a three-dose design (low, mid, and high), with half-log spacing, is standard practice in subacute toxicity studies to distinguish the NOAEL from the LOAEL.

Blood collection, hematology, and biochemistry

On day 29, following a 12-h fast, the animals were anesthetized with ketamine (65 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Blood was collected via cardiac puncture into plain tubes for serum separation and K_3-EDTA tubes for hematological analysis. The serum tubes were allowed to clot at room temperature for 30 min and were then centrifuged at 3000 rpm (1500 × g) for 15 min at 4 ˚C. The supernatant was aliquoted and stored at −20 ˚C. Hematological parameters— including red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin, hematocrit, and platelet count—were measured within 2 h using an automated analyzer (Sysmex KX-21N, Japan). Serum biochemical analysis was performed using a clinical chemistry analyzer (Roche Hitachi 912, Japan) with commercial kits (Bionic Corporation, Tehran, Iran) to measure AST, ALT, ALP, total bilirubin, albumin, total protein, creatinine, uric acid, fasting blood glucose, total cholesterol, triglycerides, sodium, and potassium. All assays were performed in duplicate, with quality-control sera included in each analytical run [21].

Histopathological examination

Following blood collection, a comprehensive necropsy was performed. The liver was excised, rinsed with ice-cold saline, blotted dry, and weighed (absolute and relative weight calculated as [liver weight/body weight] ×100). Representative specimens from the right hepatic lobe (1.0×1.0×0.5 cm) were fixed in 10% neutral buffered formalin (pH 7.4) for 48 h. The tissues were processed through ascending ethanol grades (70%, 80%, 95%, and absolute ethanol), cleared in xylene, embedded in paraffin wax, sectioned at a thickness of 5 µm using a rotary microtome (Leica RM2125, Germany), and stained with Masson's trichrome. Histological evaluation was performed by two independent pathologists who were blinded to group allocation. The parameters semi-quantitatively (0 = absent, 1 = mild, 2 = moderate, 3 = severe) included congestion, hemorrhage, inflammatory cell infiltration, and hepatocellular degeneration [22].

Statistical analysis

Data were expressed as Mean±SD. Data normality was assessed using the Shapiro–Wilk test. Normally distributed data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for pairwise comparisons. Non-normally distributed parameters (creatinine, total bilirubin, potassium, triglycerides, WBC, and platelets) were analyzed using the Kruskal-Wallis test with Dunn's post-hoc correction. Histopathological scores were compared using the Kruskal-Wallis test. $P<0.05$ were considered statistically significant. All analyses were performed using SPSS software, version 22.0 (IBM Corporation, Armonk, NY, USA).

Results

Acute oral toxicity

Single oral administration of TAHE at 2000 mg/kg produced no mortality or observable clinical signs of toxicity during the 14-day observation period. Body weight progression was unremarkable, and gross organ morphology at necropsy revealed no abnormalities. Therefore, the oral LD_{50} of TAHE exceeds 2000 mg/kg, classifying the extract as Category 5 (low acute toxicity hazard) according to OECD test guideline 423 [10].

Sub-acute toxicity: clinical signs and body weight

Repeated oral administration of TAHE (75, 150, and 300 mg/kg) for 28 days caused no mortality or treatment-related adverse clinical signs. Food and water consumption remained comparable among the groups throughout the study. Body weight gain did not differ significantly among the experimental groups (P=0.068, one-way ANOVA; Table 1).

Serum biochemical parameters

Data distribution was assessed using the Shapiro-Wilk test. Most parameters followed a normal distribution, while creatinine, total bilirubin, potassium, triglycerides, WBC, and platelets were analyzed using non-parametric methods.

Hepatic biomarkers are presented in Table 2. AST, ALT, and ALP showed no statistically significant differences between any treatment group and the control group (all P>0.05), and all values remained within the normal physiological range for male Wistar rats. AST showed a statistically significant intergroup difference (P=0.041); however, Dunnett's T3 post hoc analysis revealed that none of the individual TAHE dose groups differed significantly from the control group (all P>0.55). Total bilirubin, albumin, and total protein levels were comparable across all groups.

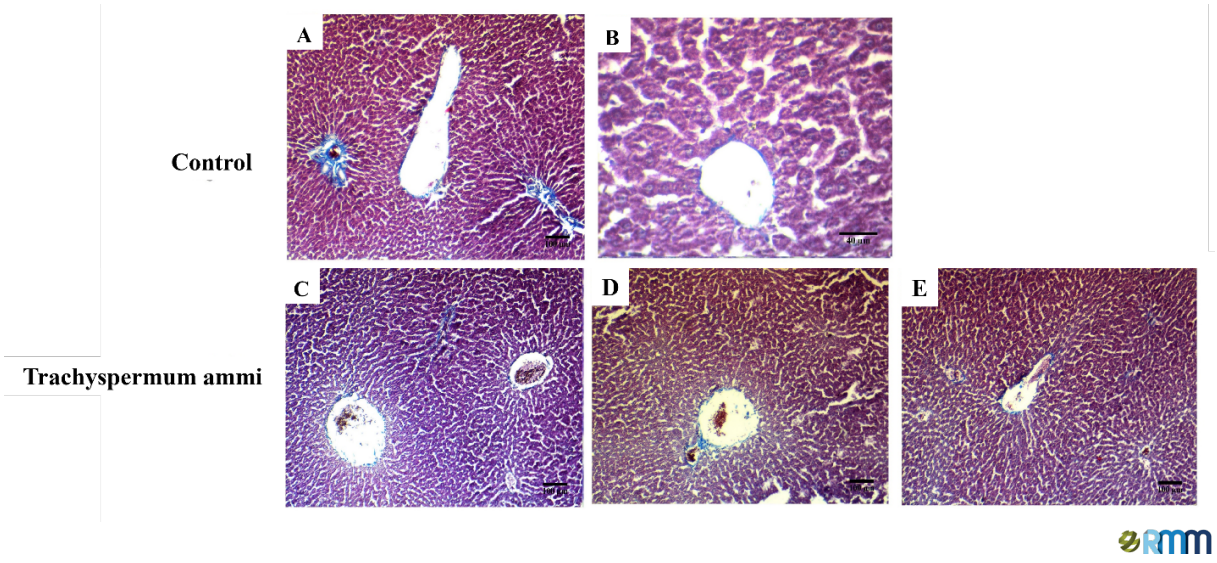


Figure 1. Masson's trichrome-stained liver sections from male Wistar rats after 28-day oral administration of TAHE

A and B) Control group: (A) portal triad and (B) central vein region showing normal hepatic architecture (×400; scale bar = 40 µm); C–E) TAHE-treated groups receiving 75, 150, and 300 mg/kg/day, respectively, showing preserved hepatocyte cords and no pathological changes (×100; scale bar = 100 µm)

Abbreviations: TAHE: *T. ammi* hydroalcoholic extract; PT: Portal triad; CV: Central vein.

Note: The kidney slides were stained using the periodic acid–Schiff technique (PAS, 100X).

Table 1. Body weight changes (g) during 28-day repeated oral administration of TAHE in male Wistar rats (n=10/group)

Group	Mean±SD					
	Day 0	Day 7	Day 14	Day 21	Day 28	Weight Gain (Day 28 – Day 0)
Control	198.2±14.3	215.6±15.1	228.9±16.2	239.5±17.8	251.3±18.4	53.1±4.2
TAHE-75	201.4±12.8	219.8±14.2	234.2±15.6	245.1±16.9	257.8±17.3	56.4±4.5
TAHE-150	199.7±13.5	217.3±14.8	231.5±15.9	242.8±17.1	254.6±17.9	54.9±4.4
TAHE-300	200.9±11.9	218.9±13.7	233.1±15.2	244.7±16.5	256.9±17.5	56.0±5.6
P*	0.87	0.91	0.86	0.82	0.79	0.81

TAHE: *T. ammi* hydroalcoholic extract.

*One-way ANOVA; P>0.05: No significant differences among groups.

Metabolic and renal biomarkers are displayed in Table 3. Total cholesterol showed a significant intergroup difference (P=0.027); however, post hoc analysis did not identify any TAHE group as significantly different from the control group. Triglyceride and fasting blood glucose levels remained unchanged. Serum uric acid levels exhibited a dose-dependent increase, reaching statistical significance at the 300 mg/kg dose compared with the control group (P=0.009, Tukey post-hoc test).

Hematological parameters

Hematological assessment revealed no significant differences in WBC count, hemoglobin, hematocrit, or platelet count among the experimental groups (Table 4). RBC count demonstrated a borderline significant intergroup difference (P=0.048); however, this difference did not persist in post hoc pairwise comparisons with the control group (Dunnett's T3 test, all P>0.05). All hematological values remained within the normal reference ranges for Wistar rats.

Organ weights and gross morphology

Macroscopic examination of the liver at necropsy revealed no treatment-related pathological findings—including discoloration, nodularity, or mass lesions—in any experimental group. Absolute and relative liver weights did not differ significantly among the groups (Table 5).

Hepatic histopathology

Histopathological examination of Masson's trichrome-stained liver sections from all groups revealed preserved hepatic architecture with intact lobular organization (Figure 1). Central veins, portal triads, and hepatic sinusoids appeared morphologically normal (Figure 1). Hepatocytes exhibited uniform cytoplasmic and nuclear characteristics with no evidence of steatosis, ballooning degeneration, Mallory-Denk bodies, or ground-glass inclusions. No significant differences in congestion, hemorrhage, inflammatory cell infiltration, or hepatocellular

Table 2. Serum hepatic function biomarkers following 28-Day oral administration of TAHE in male Wistar rats (n=10/group)

Group	Mean±SD				
	AST (IU/L)	ALT (IU/L)	Total Bilirubin (mg/dL)	Albumin (g/dL)	Total Protein (g/dL)
Control	91.2±7.8	59.8±6.2	0.31±0.04	2.91±0.13	6.82±0.31
TAHE-75	112.2±16.3	56.3±6.7	0.33±0.04	3.01±0.15	6.95±0.28
TAHE-150	121.5±18.4	57.0±7.2	0.26±0.04	3.60±0.25	7.12±0.35
TAHE-300	75.3±3.0	52.2±2.2	0.21±0.02	3.13±0.07	6.78±0.19
P*	0.041†	0.540	0.117	0.069	0.320

Abbreviations: TAHE: *T. ammi* hydroalcoholic extract; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

*One-way ANOVA. †AST: P=0.041 using ANOVA.

Table 3. Serum metabolic and renal function biomarkers following 28-day oral administration of TAHE in male Wistar rats (n=10/group)

Group	Mean±SD					
	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)	Fasting Blood Glucose (mg/dL)	Uric Acid (mg/dL)	BUN (mg/dL)	Creatinine (mg/dL)
Control	98.8±3.7	58.7±6.0	94.3±3.0	0.68±0.14	18.5±2.1	0.42±0.05
TAHE-75	92.5±6.4	56.5±7.3	89.3±2.4	0.85±0.11	19.2±1.8	0.44±0.06
TAHE-150	104.0±5.4	58.2±4.9	87.7±2.9	1.16±0.15	18.9±2.3	0.41±0.04
TAHE-300	98.7±2.2	68.0±12.1	88.3±1.5	1.41±0.21	19.8±2.5	0.45±0.07
P*	0.027†	0.230	0.210	0.009	0.680	0.550

Abbreviations: BUN: Blood urea nitrogen; TAHE: *T. ammi* hydroalcoholic extract; BUN: Blood urea nitrogen.
*One-way ANOVA with Tukey post hoc test. P = 0.009 vs control (Tukey post-hoc). †Total cholesterol: ANOVA P=0.027; post hoc analysis showed that no individual group differed significantly from the control group.

degeneration scores were observed among the groups (Table 6; all P>0.35, Kruskal-Wallis test).

Discussion

The present investigation constitutes the first systematic toxicological evaluation of TAHE, integrating acute and sub-acute oral toxicity protocols in accordance with OECD test guidelines. TAHE has a favorable safety profile, with an oral LD_{50} exceeding 2000 mg/kg, preserved hepatic architecture on histopathological examination, and no clinically significant alterations in hematological or hepatic biochemical parameters at doses up to 150 mg/kg/day administered for 28 consecutive days.

Acute toxicity and general safety

The acute toxicity study established that a single oral dose of 2000 mg/kg of TAHE produced no mortality or observable clinical toxicity. According to the Globally Harmonized System (GHS) of classification, substances with oral LD_{50} values exceeding 2000 mg/kg are classified as Category 5, indicating a low acute toxicity hazard [10]. This finding aligns with the extensive traditional use of *T. ammi* seeds as a dietary spice and household remedy, with no documented acute adverse events [14]. The absence of acute toxicity is consistent with a previous report of an oral LD_{50} above 3000 mg/kg for a standardized *T. ammi* fruit extract in rats [23].

Table 4. Hematological parameters following 28-day oral administration of TAHE in male Wistar rats (n=10/group)

Parameter	Mean±SD				P*
	Control	TAHE-75	TAHE-150	TAHE-300	
RBC (×10⁶/µL)	8.56±0.09	8.26±0.09	8.39±0.13	8.09±0.09	0.048†
Hemoglobin (g/dL)	14.91±0.72	14.58±0.48	14.77±0.55	14.35±0.39	0.546
Hematocrit (%)	43.91±1.98	42.66±2.13	43.38±1.63	42.30±1.27	0.480
MCV (fL)	51.8±2.4	52.1±2.1	51.5±2.8	52.4±2.6	0.712
MCH (pg)	17.5±0.8	17.8±0.9	17.4±0.7	17.9±1.0	0.635
MCHC (g/dL)	33.9±1.2	34.2±1.1	34.1±1.3	33.8±1.0	0.820
WBC (×10³/µL)	7.54±1.45	9.60±3.43	7.11±2.92	7.14±2.41	0.449
Platelets (×10³/µL)	835±114	893±182	901±97	886±102	0.508

Abbreviations: RBC: Red blood cells; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cells; TAHE: *T. ammi* hydroalcoholic extract.
*One-way ANOVA. †RBC: ANOVA borderline (P=0.048); Dunnett T3 post-hoc test: No individual group differed significantly from the control group (all adjusted P>0.05).

Table 5. Absolute and relative liver weights following 28-day oral administration of TAHE in male Wistar rats (n=10/group)

Group	Mean±SD		
	Final Body Weight (g)	Absolute Liver Weight (g)	Relative Liver Weight (%)
Control	251.3±18.4	9.22±1.87	3.65±0.41
TAHE-75	257.8±17.3	9.81±1.28	3.51±0.40
TAHE-150	254.6±17.9	9.83±2.34	3.35±0.55
TAHE-300	256.9±17.5	13.15±2.92	4.27±1.11
P*	0.85	0.091	0.092

TAHE: *T. ammi* hydroalcoholic extract.

*One-way ANOVA; P>0.05: no significant differences among the groups. Relative liver weight = (absolute liver weight / final body weight) ×100.

To place the present findings in a translational context, the HED was calculated according to FDA guidance using the body surface area conversion formula: HED (mg/kg) = Animal dose (mg/kg) × (Animal Km / Human Km), where rat Km = 6 and human Km = 37. The NOAEL of 300 mg/kg/day in rats corresponds to an HED of approximately 48.6 mg/kg/day, or approximately 3,406 mg/day for a 70-kg adult. Traditional consumption of *T. ammi* seeds is estimated at 1–5 g of dry seeds per day, corresponding to approximately 100–750 mg of crude extract per day, assuming an approximate extract yield of 10–15%, or approximately 1.4–10.7 mg/kg/day. Comparison of the NOAEL-derived HED (~48.6 mg/kg/day) with the estimated traditional intake (~1.4–10.7 mg/kg/day) yields a preliminary margin of safety of approximately 4.5× to 35×. However, this calculation assumes linear dose scaling, does not account for inter-species differences in absorption, metabolism, or protein binding, and should be interpreted as exploratory rather than as definitive clinical evidence.

Hepatic safety assessment

The liver was selected as the primary target organ for detailed evaluation based on its central role in xenobiotic metabolism and its recognized susceptibility to herbal-induced injury [4, 8]. The comprehensive hepatic assessment—encompassing serum biomarkers, organ weight, and histopathology—provides convergent evidence supporting the hepatic safety of TAHE under the conditions tested.

Table 6. Semi-quantitative histopathological scoring of the liver tissue following 28-day oral administration of TAHE in male Wistar rats (n=10/group)

Histopathological Finding	Mean±SD				P*
	Control	TAHE-75	TAHE-150	TAHE-300	
Sinusoidal congestion	0.11±0.33	0.14±0.37	0.16±0.40	0.33±0.51	0.352
Hemorrhage	0.00±0.00	0.28±0.48	0.16±0.40	0.16±0.40	0.358
Inflammatory cell infiltration	0.33±0.70	0.57±0.78	0.33±0.51	0.50±0.83	0.711
Hepatocyte degeneration	0.00±0.00	0.14±0.37	0.00±0.00	0.16±0.40	0.513
Necrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.000
Steatosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.000
Fibrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.000

TAHE: *T. ammi* hydroalcoholic extract.

*Kruskal-Wallis non-parametric test; P>0.05 for all parameters.

Note: Scoring system: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. All groups displayed normal hepatic architecture, with no significant pathological alterations.

Serum aminotransferases represent the cornerstone of hepatotoxicity screening in both preclinical and clinical settings [5]. In the present study, ANOVA showed a statistically significant intergroup difference in AST; however, this signal did not correspond to any biologically meaningful pattern of hepatocellular injury. Specifically, no individual TAHE group differed from the control group in post hoc analysis; the mean AST value at the highest dose (75.33 IU/L) was numerically lower than that of the control group (91.16 IU/L), and ALT—a more liver-specific aminotransferase—remained unchanged across all dose levels [6]. The dissociation between ANOVA significance and post hoc confirmation, combined with the absence of ALT elevation, indicates that the observed AST fluctuation represents Type I statistical error rather than genuine hepatotoxicity. This interpretation is consistent with established guidelines from regulatory agencies emphasizing that statistically significant aminotransferase elevations lacking dose-response relationship and histological correlation should not be considered toxicologically significant [24].

The preservation of hepatic synthetic function, evidenced by unchanged serum albumin and total protein concentrations, provides additional evidence against clinically significant hepatocellular damage. Albumin, with a half-life of approximately 20 days in rats, serves as a reliable indicator of chronic hepatic functional capacity during 28-day toxicity studies [7]. The absence of hypoalbuminemia across all TAHE-treated groups indicates that prolonged extract administration did not impair hepatic protein synthesis.

Histopathological correlation

Histopathological examination represents the reference standard for characterizing hepatic injury, providing spatial and morphological information not captured by serum biomarkers [25]. The preservation of normal hepatic architecture—with intact central veins, unremarkable portal tracts, and hepatocytes maintaining normal polygonal morphology—provides definitive evidence against structural hepatotoxicity. The application of Masson's trichrome staining, which specifically highlights collagen deposition, confirmed the absence of early fibrotic changes that might precede biochemical abnormalities [26]. The mild, scattered inflammatory foci observed across all groups including the control group, are consistent with background incidental findings commonly encountered in laboratory rodents and do not constitute evidence of treatment-related pathology [22].

Serum uric acid elevation: A finding requiring attention

The dose-dependent elevation in serum uric acid, which reached statistical significance at 300 mg/kg (P=0.009), represents the sole biochemical alteration attributable to TAHE administration. Uric acid is the terminal product of purine metabolism in humans and higher primates; however, in rats, endogenous uricase converts uric acid to allantoin, resulting in substantially lower basal serum urate concentrations compared to humans [27]. Consequently, uric acid elevations in rodent models require careful interpretation.

Several non-mutually exclusive mechanisms could account for this observation. First, thymol and related phenolic monoterpenes have been reported to inhibit xanthine oxidase, the enzyme catalyzing the terminal steps of purine catabolism; paradoxical uric acid elevation could reflect alterations in renal urate handling rather than increased production [28]. Second, flavonoids present in the hydroalcoholic extract may compete with uric acid for renal tubular secretion via organic anion transporters (OAT1/OAT3), thereby reducing urate clearance [29]. Third, the ethanol component of the extraction solvent (70%) may have extracted compounds that subtly modulate purine metabolism. This hypothesis warrants targeted investigation in subsequent studies, including the measurement of urinary uric acid excretion and renal urate transporter expression.

Despite its statistical significance, the clinical relevance of the uric acid elevation at 300 mg/kg requires cautious interpretation. The magnitude of elevation (approximately 2-fold) remained moderate; no histopathological evidence of urate crystal deposition or tubulointerstitial nephropathy was observed, and renal function parameters (BUN [blood urea nitrogen] and creatinine) remained within normal limits [30]. Nevertheless, this finding underscores the importance of monitoring uric acid in longer-duration studies and informed the conservative assignment of the NOAEL.

Determination of NOAEL

Based on the integrated analysis of all endpoints, a NOAEL of 150 mg/kg/day is proposed for the 28-day sub-acute study. This determination reflects the convergence of evidence from hematological, biochemical, and histopathological assessments: at 150 mg/kg, no parameter exhibited toxicologically significant alteration from control values. The 300 mg/kg dose, although not associated with histopathological or hematological abnor-

malities, resulted in a statistically significant elevation in serum uric acid, precluding its assignment as a no-effect level. Extrapolation using a standard uncertainty factor (UF=100) suggests an acceptable daily intake (ADI) of approximately 1.5 mg/kg/day for humans, consistent with the quantities typically consumed through dietary intake of ajwain as a spice [12].

Limitations of the study

Several limitations of the present investigation should be acknowledged to facilitate accurate interpretation and guide future research directions. First, although the 28-day subacute study duration complied with OECD 407 guidelines, it does not address the possibility of cumulative toxicity or carcinogenicity, which would require subchronic (90-day) or chronic (6–12-month) exposure protocols [11]. Second, the absence of toxicokinetic data, including plasma concentration–time profiles of major constituents such as thymol, precludes correlating the administered doses with systemic exposure. Third, the isolated uric acid elevation at 300 mg/kg was assessed only at the terminal time point; serial measurements and urinary excretion data would strengthen mechanistic interpretation. Fourth, comprehensive urinalysis, including urinary sediment examination, was not performed, limiting the assessment of potential renal effects. Fifth, the study did not include a satellite recovery group to assess the reversibility of the observed uric acid elevation after cessation of extract administration [22]. Finally, phytochemical characterization was limited to qualitative confirmation of the extraction method; quantitative standardization of the extract to specific marker compounds (e.g. thymol content) would enhance inter-study reproducibility.

Conclusion

The present investigation establishes that TAHE possesses a favorable acute and sub-acute oral safety profile in male Wistar rats. The oral LD_{50} exceeds 2000 mg/kg, classifying the extract as a low acute toxicity hazard. The unchanged aminotransferase levels, preserved hepatic synthetic function, normal liver weight, and intact hepatic architecture on histopathological examination collectively provide robust evidence of hepatic safety under the conditions tested. A NOAEL of 150 mg/kg/day is proposed, with the dose-dependent uric acid elevation at 300 mg/kg/day identified as the dose-limiting finding. These data provide essential toxicological reference values supporting the traditional use of *T. ammi* and establish the safety foundation necessary for future preclinical pharmacological investigations and rational dose selection.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was approved by the Institutional Animal Ethics Committee of Birjand University of Medical Sciences, Birjand, Iran (Code: IR.BUMS.REC.1396.17), and all procedures followed institutional guidelines for the ethical care and use of laboratory animals.

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Author's contributions

Conceptualization and study design, and supervision: Davood Dorranipoor; Data acquisition: Davood Dorranipoor and Reza Dehghani; Analysis and data interpretation: Iman Zangiabadi, Mostafa Peyvandi; Statistical analysis: Iman Zangiabadi and Mohammad Zangooei Writing the original draft: Mostafa Peyvandi; Review and editing: Mostafa Peyvandi and Mohammad Zangooe; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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