

Pharmacokinetics and Toxicity Study of a Single Intravenous Dose of Distilled Extract of *Zanthoxylum piperitum* in ICR Mice

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Abstract

This study aimed to investigate the pharmacokinetics and acute toxicity of a single intravenous dose of distilled *Zanthoxylum piperitum* (deZP) extract in ICR mice. The focus was on understanding the absorption, distribution, and elimination of Terpinen-4-ol, the major active component of deZP, as well as assessing its safety at the administered dose. ICR mice were administered a single intravenous dose of deZP (2.4 mg/kg), and plasma concentrations of Terpinen-4-ol were measured using LC-MS/MS at various time points. Pharmacokinetic parameters such as C_{max}, T_{max}, half-life, and clearance rate were calculated. In addition, clinical signs, mortality, and biochemical markers were monitored to evaluate acute toxicity. Terpinen-4-ol was rapidly absorbed, reaching a peak plasma concentration (C_{max}) of 609.0 ng/mL within 0.083 hours (T_{max}). It exhibited a short half-life of 0.168 hours and a high clearance rate of 194.1 L/h/kg. No mortality or significant clinical signs of toxicity were observed. Biochemical parameters, including liver and kidney function markers, remained within normal ranges, indicating that the administered dose was well-tolerated. The pharmacokinetic profile of deZP suggests rapid absorption and elimination of Terpinen-4-ol, while the toxicity assessment indicates good tolerability at the tested dose in ICR mice. These findings provide a foundation for further exploration of deZP's therapeutic potential.

1. Introduction

Zanthoxylum piperitum (ZP), commonly known as Japanese pepper, is a traditional medicinal plant that has been extensively utilized in East Asia, particularly in countries like Korea, China, and Japan. ZP is frequently employed as a spice, in traditional medicine, and for oil refinement due to its abundance of spices and active oil components found in its roots, stems, leaves, and fruits. The distilled extract of ZP (deZP) comprises various compounds including myrcene, octanal, d-limonene, linalool, citronellal, geraniol, phellandral, and geranyl acetate (1, 2). The primary constituents of deZP can vary depending on the extraction source and method. Choochote et al. identified hydrocarbons and alcohols, mainly d-limonene, sabinene, and β -myrcene, as the predominant volatile components in the fruit of ZP (3). Meanwhile, Abreu et al. found that germacrene D and bicyclogermacrene were the key components in the essential oil from the leaves, while the fruits primarily contained menth-2-en-1-ol, beta-myrcene, (-)-linalool, and (-)-alpha-terpineol. Additionally, beta-myrcene and menth-2-en-1-ol were the dominant compounds in the essential oil of the flowers (4).

ZP is well-known for its wide range of pharmacological activities, including anti-inflammatory, antibacterial, antioxidant, and anti-allergic properties (5–11). The anti-inflammatory effects of ZP are largely attributed to its ability to inhibit pro-inflammatory cytokines and mediators such as Tumor necrosis factor- α (TNF- α), Interleukin 6 (IL-6), and nitric oxide (NO) (12, 13). Additionally, ZP extracts have been shown to inhibit the activation of NF- κ B and MAPK signaling pathways, which play key roles in the inflammatory response (1, 13). Despite the benefits, there is a lack of extensive research on the pharmacokinetics (PK) and toxicity of ZP extracts in animal models like mice or rats. Investigating the PK and potential toxicity of ZP extracts is essential to validate their safety and effectiveness for medicinal use.

In this study, we identified Terpinen-4-ol as the major constituents of deZP (14, 15), and evaluated its pharmacokinetics and single-dose toxicity in ICR mice. By analyzing the PK profile and assessing the single-dose toxicity of deZP, this research aims to provide a thorough evaluation of its therapeutic potential and safety, thereby supporting the development of deZP-based functional foods, nutraceuticals, and pharmaceuticals.

2. Materials and Methods

2.1. Chemicals and reagents

Distilled extracts of *Zanthoxylum piperitum* (deZP) was produced that mixed with 10 liters distilled water in extractor and boil at 105°C for 2 hours. ZP extracts performed distilled extract about 5 liters water at 107 °C. The completed deZP was stored in 4 °C. Terpinen-4-ol, tolbutamide and DMSO purchased from Sigma-Aldrich and acetonitrile, methanol and water were from Burdick & Jackson (MI, USA). Formic acid was Fisher Chemical. All solvents and chemicals were of analytical grade.

2.2. Animals

The animals used in this study were 7-week-old ICR (CrI/Ori: CD1) mice (Koatech Inc., Gyeonggi-do, Korea). The mean weights of the 40 mice were 27.4 ~ 34.4 g. The mice were housed in a controlled animal facility with a 12:12-h light/dark cycle from 7 AM to 7 PM at a 150–300 lux illumination intensity, a temperature of 23 ± 3°C, and a relative humidity of 30–70%. Commercial mouse chow (SAFE + 40) was put in feeders and provided ad libitum. Water (UV-irradiated filtered tap water) was also provided ad libitum. All mice were dosed after an overnight fast except for water. The Institutional Animal Care and Use Committee (IACUC) of Woojung Bio Inc. (Gyeonggi-do, Korea) approved the experimental procedures and animal care (IACUC 2303-042).

For the plasma samples, a 20 µL plasma (or blank plasma for method validation of reference terpinen-4-ol standard solution) was successively added with 80 µL blank methanol or 80 µL 1mg/mL IS solution. After vortexing for 60 seconds, the samples were centrifuged at 15,520 g for 10 minutes. Then, 5 µL of the supernatant liquid was injected into the LC-MS/MS system for analysis.

2.3. Validation for Calibration curves and low limit of quantitation (LLOQ)

We prepared the calibrators by diluting each with 0.1–100.0 µg/mL terpinen-4-ol in methanol. The stock solutions were added to blank plasma to provide around 0.005–5.0 µg/mL terpinen-4-ol. The calibration curve was obtained by plotting the peak area ratio of [terpinen-4-ol/IS] as a function of the respective concentrations of terpinen-4-ol, and calculating the linear regression.

Determining the low limit of quantitation (LLOQ) was conducted to approach the lowest concentration on the calibration curve in which precision and accuracy were within ± 20% (Guidance for Industry, Bioanalytical Method Validation, 2001), the LOQ was accounted for the final concentration producing a signal to noise ratio more than 5 and evaluated by using eight independent samples.

2.4. Pharmacokinetic study

The pharmacokinetic study aimed to determine the T_{max} and C_{max} of terpinen-4-ol after intravenous (i.v.) administration of the deZP (2.4 mg extract/kg body weight). Forty mice were randomly separated into 8 groups based on predetermined time points, with five mice at each time point (Table 1). deZP was administered intravenously (i.v.) in a concentration of 2.4 mg/kg body weight ($n = 5$). Blood samples were obtained by cardiac puncture according to specific time schedules of 0.083, 0.25, 0.5, 1, 2, 4, 8 and 24 h. EDTA-anticoagulated whole blood samples were centrifuged at 1000 rpm for 10 minutes. The supernatant layer of the blood was collected as plasma and stored at -80°C until LC-MS/MS assay.

The pharmacokinetic model and parameters were calculated using PKSolver. The maximal plasma concentration (C_{max}) was derived directly from the experimental data. Key pharmacokinetic parameters were calculated through noncompartmental analysis. The area under the plasma concentration–time curve from time zero to the last measurable time point ($AUC_0\text{--}AUC_{last}$) was determined using the linear trapezoidal rule. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\text{Beta}$. Systemic clearance (CL_F) was calculated as $\text{Dose}/AUC_0\text{--}\infty$, and the apparent volume of distribution ($V1_F$) was determined as CL_F/Beta .

2.5. Biochemical analysis

Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, bilirubin, alkaline phosphatase (ALP), albumin, total cholesterol (TC), HDL-cholesterol and triglycerides (TG) were determined using an enzymatic assay kit (Asan Pharm, Seoul, South Korea) according to the manufacturer's instructions.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Statistical significance was evaluated by one-way analysis of variance (ANOVA) with Tukey's post hoc test. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Method validation

Terpinen-4-ol is a terpeneol that is 1-menthene carrying a hydroxy substituent at position 4 (Fig. 2A-E). It has a role as a plant metabolite, an antibacterial agent, an anti-inflammatory agent, an antiparasitic agent, an antineoplastic agent, an apoptosis inducer and a volatile oil component. Tolbutamide was used as internal standard (IS) to determine terpinen-4-ol in mouse plasma.

MRM chromatograms of mouse plasma spiked with 5.0 ng/mL (LLOQ, lower limit of quantitation) of terpinen-4-ol and IS (Fig. 2F-I). Calibration samples were prepared using mouse blank plasma, which were spiked with terpinen-4-ol at known concentrations (5.0–5000.0 ng/mL). The quadratic regression evaluation of the relationship of peak area ratios (terpinen-4-ol/IS) (y) versus terpinen-4-ol concentration (x) was $y =$

$0.004661x^2 + 0.201455x + 0.001980$. The coefficient of correlation (r) was 0.99993. The matrix effect and recovery yield were all within $\pm 15\%$, which was generally accepted to carry out the analysis of biological samples containing the drug. In summary, these method validation results confirmed that our subsequent analyses were accurate and reliable.

3.2. Pharmacokinetic study

The plasma mean concentration–time curves of terpinen-4-ol in ICR mice after administration of 2.4 mg/kg deZP is shown in Fig. 3 and Table 1. These data demonstrated that terpinen-4-ol was absorbed and eliminated relatively quickly from the plasma. Using the PKSolver 2.0 software, the pharmacokinetic model and parameters were calculated to provide a detailed understanding of the compound's disposition in vivo.

The main PK parameters of Terpinen-4-ol in ICR mice following intravenous injection are summarized in Table 1. The time to reach maximal plasma concentration (T_{max}) was observed at 0.083 hours, with the peak plasma concentration (C_{max}) reaching 609.0 ng/mL. The area under the plasma concentration-time curve from zero to the last measurable concentration (AUC_{0-last}), calculated using the trapezoidal rule, was found to be 203.3 h·ng/mL. The clearance rate was determined to be 194.1 L/h/kg, indicating a relatively rapid elimination from the systemic circulation. The terminal half-life ($t_{1/2}$) of Terpinen-4-ol was 0.168 hours, reflecting its quick elimination from the plasma. Notably, Terpinen-4-ol was detectable in the mouse plasma up to 1 hour post-administration, suggesting a short duration of systemic exposure.

These pharmacokinetic characteristics suggest that Terpinen-4-ol, the major component of deZP, is rapidly absorbed and cleared in mice, which could influence its pharmacodynamic effects and therapeutic potential. The rapid absorption and elimination might require consideration of dosing frequency in potential therapeutic applications. Further studies could explore the impact of repeated dosing and the pharmacokinetics in other animal models to better understand the implications of these findings in broader contexts.

Table 1
Pharmacokinetic parameters of terpinen-4-ol after injection in ICR mice.

Parameters	unit	value
AUC _{0–last}	ng/mL·h	203.301
AUC _{0–∞}	ng/mL·h	206.054
MRT _∞	h	0.204
C _{max}	ng/mL	609.015
T _{max}	H	0.083
CL(obs)	mL/min/Kg	194.124
V _{ss} (obs)	L/Kg_IV	2.375
V _z (obs)	L/Kg_IV	2.824
t _{1/2}	h	0.168
AUC _{0–last} : AUC from time zero to the last time point; AUC _{0–∞} : AUC with extrapolation to infinity; MRT, mean retention time; C _{max} , maximal plasma concentration; T _{max} , highest temperature; CL, clearance; V _{ss} , Volume of distribution at steady state; V _z , Volume of distribution; t _{1/2} , terminal half-life		

3.3. Mortality and clinical signs

Throughout the observation period, no mortalities were recorded in the all group administered with 2.4 mg/kg of deZP. All animals appeared healthy and exhibited no significant clinical signs of toxicity, including abnormal behaviors, changes in body posture, or signs of distress. The results are summarized in Table 4. The absence of adverse effects and mortality in the deZP-treated group suggests that the single intravenous dose of 2.4 mg/kg is well-tolerated in ICR mice. This finding provides preliminary evidence supporting the safety profile of deZP at the tested dose, paving the way for further studies on its therapeutic potential.

3.4. Biochemical analysis in mouse plasma

Following the completion of the single-dose survival rate test with deZP, blood biochemical analyses were performed on serum samples collected at the point of sacrifice. The results of these tests are summarized in Table 4. The analysis included measurements of key biochemical indices such as liver enzymes (ALT, AST), kidney function markers (creatinine, BUN), and other relevant serum components.

The levels of all measured indices were found to be within their respective normal ranges, indicating no significant deviations from the baseline. Moreover, no biochemical alterations attributable to deZP administration were observed, suggesting that deZP does not induce any detectable acute toxicological effects in these parameters at the administered dose. These findings imply that the single-dose administration of deZP is biochemically well-tolerated in ICR mice, with no adverse effects on liver or kidney function, or other vital biochemical markers under the conditions of this study (Table 2).

Table 2
Biochemical analysis of serum from mice injected with deZP via tail vein.

AST (IU/L)	ALT (IU/L)	BUN (mg/dL)	Cr (mg/dL)	Bilirubin (mg/dL)	ALP (K-A)	Albumin (g/dL)	TC (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
25.9 ± 0.11	25.7 ± 0.27	9.8 ± 0.38	0.81 ± 0.031	0.44 ± 0.015	5.4 ± 0.79	4.1 ± 0.28	145.4 ± 9.33	51.5 ± 0.65	138.4 ± 18.42

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; ALP, alkaline phosphatase; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride

4. Discussion

This study aimed to evaluate the pharmacokinetics and toxicity of a single intravenous dose of deZP in ICR mice. The primary component of the extract, Terpinen-4-ol, was selected as the standard for quantification due to its predominance in our specific extraction protocol. The pharmacokinetic analysis revealed that Terpinen-4-ol was rapidly absorbed and eliminated in the plasma, with a T_{max} of 0.083 hours and a terminal half-life of 0.168 hours. The clearance rate was relatively high, at 194.1 L/h/kg, suggesting a swift systemic elimination of the compound. These results indicate that Terpinen-4-ol, and consequently deZP, may have a short duration of action in vivo, which could influence its pharmacodynamic properties and necessitate frequent dosing in therapeutic applications.

Terpinen-4-ol is known for its various pharmacological activities, including antibacterial, anti-inflammatory, and anticancer properties (16–20). Studies have shown that Terpinen-4-ol exhibits significant antimicrobial effects against a range of bacterial and fungal pathogens, making it a valuable component in therapeutic formulations aimed at treating infections (19, 21). Additionally, its anti-inflammatory activity has been demonstrated in various models, where Terpinen-4-ol reduced the production of pro-inflammatory cytokines and alleviated symptoms of inflammation (17, 22, 23). The compound also shows potential in cancer therapy, with studies indicating its ability to induce apoptosis in cancer cells and inhibit tumor growth (18, 24). As mentioned earlier, ZP is well-regarded for its broad spectrum of pharmacological activities, particularly its anti-inflammatory, antibacterial, and anti-allergic properties (5–11). The pharmacological effects of Terpinen-4-ol, which are similar to those of ZP, suggest that the observed effects of deZP may be due to the presence of Terpinen-4-ol.

Toxicological assessment was conducted through mortality observations, clinical signs, and biochemical analyses of serum samples. Throughout the study, no mortalities were recorded in any of the deZP-treated groups, and no significant clinical signs of toxicity were observed. Biochemical parameters, including liver enzymes (ALT, AST), kidney function markers (creatinine, BUN), and other vital indicators, remained within normal ranges, further supporting the safety of the administered dose. These findings suggest that deZP, at the tested dose of 2.4 mg/kg, is well-tolerated in ICR mice, with no evident acute toxic effects.

The rapid absorption and elimination of Terpinen-4-ol, as well as the absence of significant toxicological effects, provide valuable insights into the potential therapeutic use of deZP. However, further studies are warranted to explore the pharmacokinetics of repeated dosing, as well as the effects of higher doses, to fully understand the safety and efficacy profile of deZP. Additionally, studies in other animal models could provide broader insights into the generalizability of these findings. Understanding the pharmacodynamics in conjunction with the pharmacokinetics will be crucial in determining the appropriate therapeutic applications and dosing regimens for deZP.

In conclusion, this study provides foundational data on the pharmacokinetic behavior and safety of *Zanthoxylum piperitum* extract, highlighting its rapid elimination and good tolerability in ICR mice. Coupled with the known pharmacological effects of Terpinen-4-ol, these findings pave the way for further research into the therapeutic potential of deZP and its major constituent, Terpinen-4-ol.

5. Conclusion

The conclusion of the paper suggests that the distilled extract of *Zanthoxylum piperitum* (deZP), with Terpinen-4-ol as its primary active component, is rapidly absorbed and eliminated when administered intravenously to ICR mice. The pharmacokinetic analysis demonstrated that Terpinen-4-ol reaches a peak concentration shortly after administration and has a relatively short half-life. Importantly, no significant signs of toxicity or mortality were observed, and key biochemical markers remained within normal ranges, indicating that deZP is well-tolerated at the tested dose. These results provide initial evidence supporting the safety and potential therapeutic application of deZP, warranting further research into its efficacy and pharmacodynamic properties.

Declarations

Author Contributions

Writing—original draft preparation, J.Y.J. and J.H.L.; Writing—review and editing, visualization, G.Y., J.H.L. and J.U.K.; Conceptualization, G.Y. and T.H.Y.; funding acquisition, J.U.K.; Supervision, T.H.Y.; Design of the study, experiment, statistical analysis, and interpretation of the data, J.Y.J. and J.H.L. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest that are directly relevant to the content of this study.

Consent to Publish declaration

Not applicable.

Consent to Participate declaration

Not applicable.

Ethical statement

This research was approved by the institutional animal care and use committee (IACUC 2303-042, approval date 2024.11.08).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

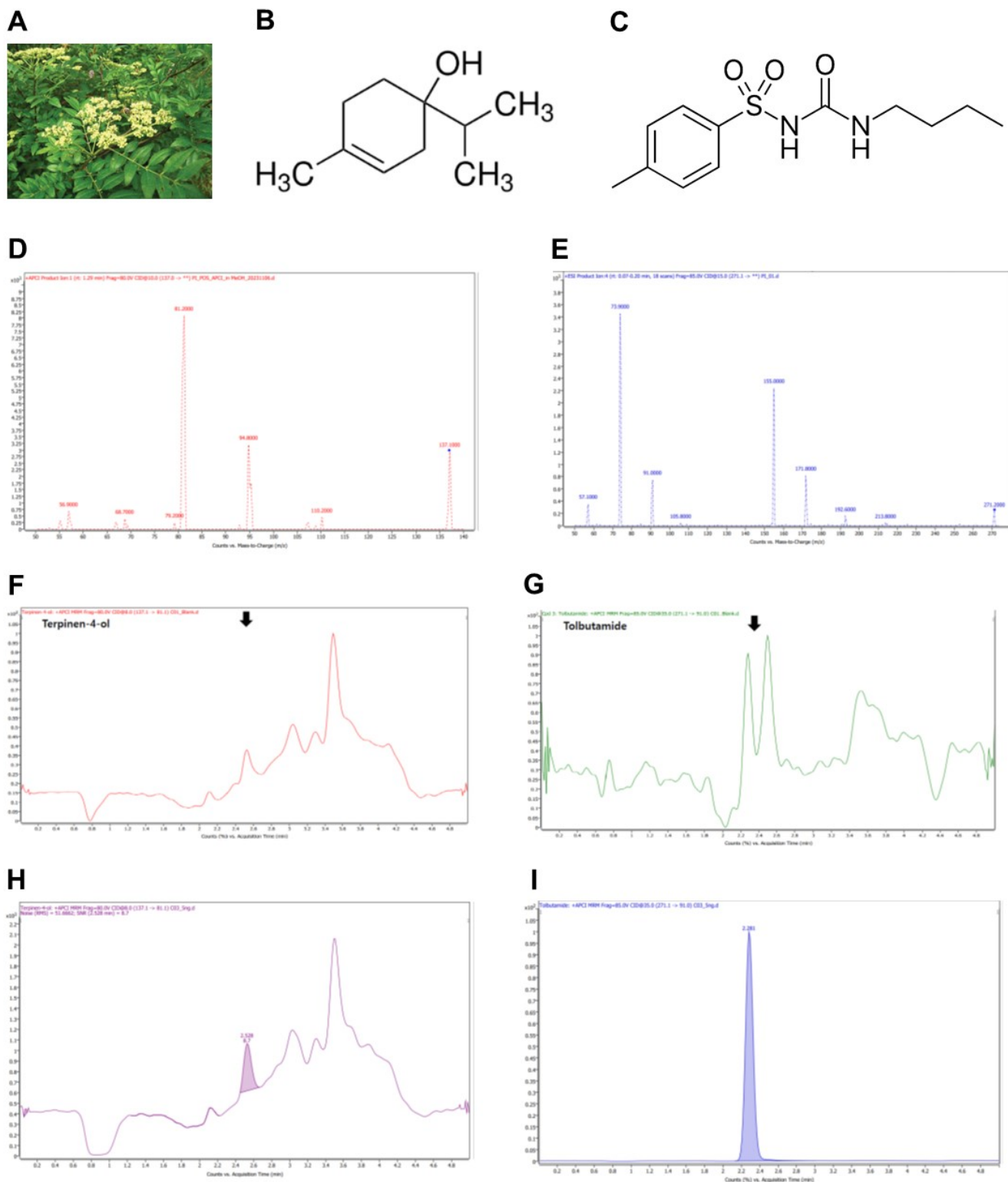
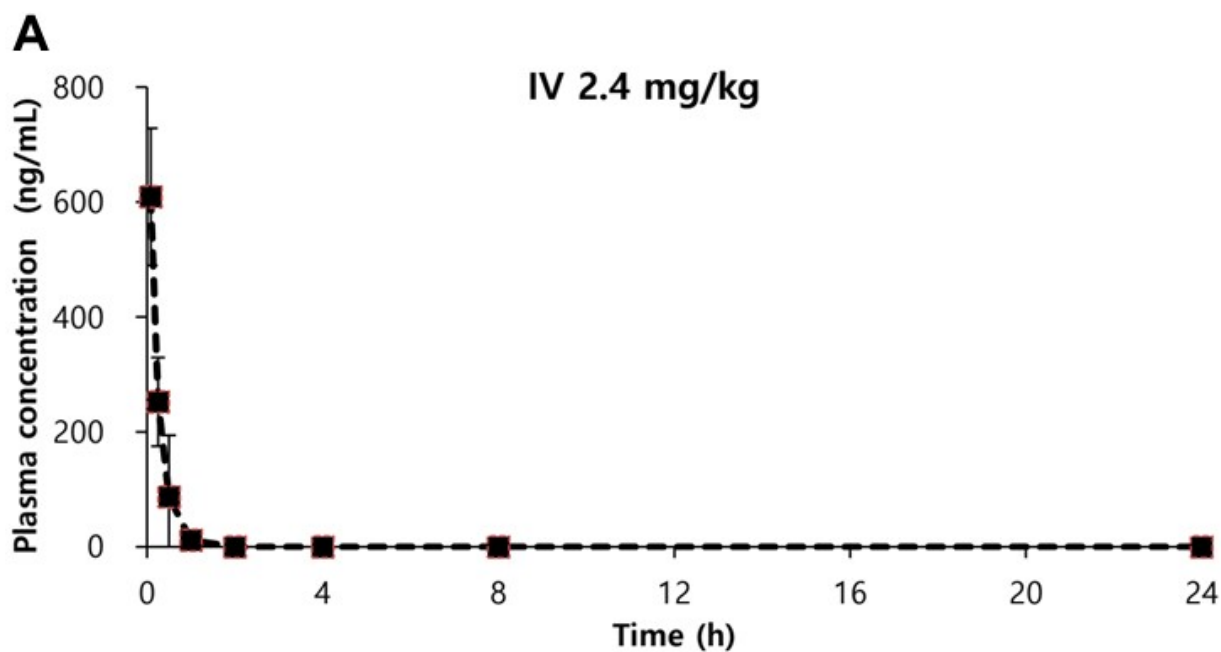


Figure 1

Zanthoxylum piperitum (A) and internal standard. Chemical structures of Terpinen-4-ol (B) and internal standard tolbutamide (C). LC-MS/MS spectrum of Terpinen-4-ol (D) and IS (E). Representative chromatogram: Blank matrix of Terpinen-4-ol (F) and IS (G). LLOQ of Terpinen-4-ol (H) and IS (I).



B

Group	Animal No.	IV Time(hr)	Concentration (unit ng/ml, n=5)	Body weight (g)
G1	5	0.083	609.015±119.253	31.5±1.9
G2	5	0.25	252.256±77.135	31.5±1.7
G3	5	0.5	86.859±107.286	30.3±1.8
G4	5	1	11.357±3.873	31.9±0.7
G5	5	2	0.000±0	32.8±1.5
G6	5	4	0.000±0	30.4±2.1
G7	5	8	0.000±0	30.0±1.4
G8	5	24	0.000±0	29.7±1.4

Figure 2

Plasma concentration versus time profile of terpinen-4-ol after intravenous injection at 2.4 mg/kg to ICR mice. Each point represents mean $\pm$ SD (n=5).