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The Evaluation of the Acute Toxicity and Safety of Verbenalin in ICR Mice

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ABSTRACT

Background: Verbenalin is an iridoid glucoside, which is among the active components of some medicinal herbs such as Verbena officinalis Linn, and Cornus officinalis Siebold and Zucc. Previous studies have confirmed the antioxidant activity and neuroprotective potential of verbenalin. To confirm the safety of verbenalin, an approximate lethal dose was determined based on a single oral dose toxicity study.
Methods: Institute of Cancer Research mice were randomly assigned to three verbenalin exposure groups (250, 500, and 1,000 mg/kg) and a control group (5% methylcellulose solution). There were (5 male and 5 female mice per group). Mortality, clinical signs, and body weight were monitored for 14 days, and necropsies were conducted.
Results: No mortalities were observed in the control group or the verbenalin 250 mg/kg group, whereas mortalities were observed in the 500 mg/kg and 1,000 mg/kg verbenalin groups. During the observation period, stool abnormalities such as mucous stools were observed. Clinical signs such as loss of locomotor activity were observed in the 500 mg/kg and 1,000 mg/kg verbenalin groups. During the study period, significant changes in body weight were observed in the 500 mg/kg and 1,000 mg/kg verbenalin groups; however, no gross abnormalities were observed at necropsy. Overall, no toxicity was found in the 250 mg/kg group.
Conclusion: The approximate lethal dose of verbenalin was estimated to be 500 mg/kg. For a more accurate assessment of the safety of verbenalin, other types of studies such as repeated-dose toxicity studies should also be conducted.

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Introduction

Historically, medicinal plants have been used for the prevention and treatment of conditions/diseases [1], and today they are still in frequent use. Despite the lack of scientific evidence on the efficacy of some medicinal plants, they are widely used for a range of conditions/diseases [2]. Medicinal plants can cause toxicity [3], therefore, research on the safety of medicinal plants should be performed.

Verbenalin, equivalent to verbena glycoside, is one of the active ingredients of Verbena officinalis Linn [4] and Cornus officinalis Siebold and Zucc [5]. Previous studies have reported that verbenalin also acts as an antioxidant, improves mitochondrial function, and protects neurons [6,7]. Among Korean medicine decoctions, there is Yukmijihwangtang using Cornus officinalis Siebold and Zucc containing verbenalin, and it is reported that Yukmijihwangtang

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can be used for the treatment of brain damage caused by cerebral ischemia [8]. Although the effects of verbenalin have been demonstrated, no toxicity tests have been conducted thus far.

For verbenalin to be used clinically, it is essential to conduct general toxicology tests for safety information. To date, toxicity studies have been conducted on Verbena officinalis and Corni fructus (dry ripe fruits of Cornus officinalis Siebold and Zucc), but no toxicity studies with verbenalin alone.

Materials and Methods

Test substance

Verbenalin (purity > 99.3%, molecular formula: C17H24O10, molecular weight: 388.37; Chengdu Biopurity Phytochemicals Ltd., Chengdu, China) was prepared using 0.5% methylcellulose (MC) solution, and MC 1,500 cP (Junsei Chemical Co. Ltd., Tokyo, Japan) dissolved in water for injection (JW Pharmaceutical Co. Ltd., Seoul, Korea). Experimental solutions were then made up to the desired concentrations.

Experimental animals

The animals used in this study were Institute of Cancer Research (CrlOri: CD1) mice (Orient Bio Inc., Gyeonggi-do, Korea). These mice were selected because they are widely used in toxicity studies and there is an abundance of relevant data on these animals. There were 24 males weighing 24.8–28.2 g and 24 females weighing 18.9–22.6 g who were all 5 weeks old. Three mice were housed in stainless steel wire mesh cages during the quarantine-acclimatization period, after which only one animal was housed per cage during the observation period. The temperature in the laboratory was 21.3–23.9°C, the relative humidity was 54.0–63.5%, the air was exchanged 10–15 times per hour, and the mice were kept at a 12 hour/12-hour light/dark cycle from 7 AM to 7 PM at a 150–300 lux illumination intensity. Commercial mouse chow (Teklad Certified Irradiated Global 18% Protein Rodent Diet) was put in feeders and provided ad libitum. Water (UV-irradiated filtered tap water) was also provided ad libitum.

Following the quarantine-acclimatization period, 1 week later, experiments were conducted on 20 males weighing 28.0–32.0 g and 20 females weighing 20.5–23.5 g. All experiments in this study were conducted at Biotoxtech Co., Ltd. and in compliance with the Animal Protection Act of the Republic of Korea, the Guide for the Care and Use of Laboratory Animals [9]. Biotoxtech Co., Ltd. received full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International in 2010. This study was approved by the Institutional Animal Care and Use Committee of Biotoxtech Co., Ltd. based on the Animal Protection Act of the Republic of Korea (Enactment May 31, 1991, No. 4379, Revision Feb 11, 2020, No. 16977; Approval No. 210680) Furthermore, this study was conducted according to ARRIVE guidelines [10].

Acute oral toxicity study

This study was conducted following the Organization for Economic Co-operation and Development Guidelines for the Testing of Chemicals, 423 [11]. During the quarantine-acclimation period, the animals were monitored for clinical signs once a day. The animals were quarantined for 3 days in a quarantine room and then moved to the animal room. On the last day of the quarantine-acclimation period, body weights were recorded, and clinical signs and body weight changes were examined to assess the health condition of all animals. Based on these observations, it was determined that the animals had no pre-existing abnormalities. During the observation period, a unique identification number was marked on the tail of each animal, and an animal identification card was attached to each cage describing the group and dose level. On the last day of the quarantine-acclimation period (day of group assignment), 20 males and 20 females with similar body weights were selected and assigned to 4 experimental groups (5 animals/sex/group). The selected animals were randomly assigned to achieve an even distribution of the mean body weight of each group (Table 1).

In a preliminary study (Biotoxtech Study No. B21987P1, B21987P2), doses of 2,000, 1,000, and 500 mg verbenalin/10 mL/kg were each orally administered to one male and one female, respectively. On Day 2, one male exposed to the 1,000 mg/kg verbenalin dose was found dead. On Day 3, another male exposed to the 2,000 mg/kg dose was also found dead. No mortalities were observed at the 500 mg/kg verbenalin dose. Based on these preliminary results, 1,000 mg/kg verbenalin was selected as the high

Table 1. Group Designation.

Group	Dose weight (mg/kg)	Dose volume (mL/kg)	No. of animals (ID)	
			Males	Females
G1* (control)	0	10	5 (1101-1105)	5 (2101-2105)
G2† (low dose)	250	10	5 (1201-1205)	5 (2201-2205)
G3‡ (mid dose)	500	10	5 (1301-1305)	5 (2301-2305)
G4§ (high dose)	1,000	10	5 (1401-1405)	5 (2401-2405)

* Group 1 = 0mg/kg dose group.
† Group 2 = 250mg/kg dose group.
‡ Group 3 = 500mg/kg dose group.
§ Group 4 = 1,000mg/kg dose group.

dose for further experiments. By applying a geometric ratio of 2, the mid and low doses were selected at 500 mg/kg and 250 mg/kg verbenalin, respectively. Control animals were dosed with the same volume of the vehicle (0.5% methylcellulose) for verbenalin.

To assess the toxicity of oral exposure to verbenalin all treatments were administered orally via gastric intubation. The individual dose volumes for each animal were calculated based on the weight of the animals recorded in the fasting state on the day of dosing. All animals were dosed via gastric intubation with disposable syringes fitted with an intubation tube. The animals fasted for approximately 4 hours before dosing. Drinking water was provided ad libitum. Feed was provided approximately 2 hours after dosing.

Parameters evaluated

On the day of dosing (Day 1), clinical signs (type, severity, time of onset, and recovery) and mortality were monitored in all animals 30 minutes after dosing, and 1, 2, 4, and 6 hours after dosing. Afterwards, the same procedure was performed once a day for 14 days (i.e., from Day 2 to Day 15). The body weights of the animals found dead during the observation period were measured, after which the mice were immediately refrigerated until the dead animals were necropsied which was as quickly as possible. The body weight was recorded once on the day of dosing (before dosing), and on Days 2, 4, 8, and 15 (the day of necropsy). On the day of necropsy, all the animals were anesthetized by CO2 gas inhalation and exsanguinated from the abdominal aorta. A post-mortem test was performed to collectively observe all organs for all animals in the study. No visual indications of disease were observed during the necropsy, and therefore no additional histopathological tests were performed.

Statistical analysis

Statistical analyses were performed on the body weight data using the SAS program (Version 9.3, SAS Institute Inc., USA). The measurement data were analyzed using Bartlett's test for homogeneity of variance (significance level: 0.05). One-way analysis of variance was used for homogeneous data (significance level: 0.05). If significant differences were identified, multiple comparisons were conducted via Dunnett's test (significance levels: 0.05 and 0.01, two-tailed).

Results

Mortality

During the observation period, no mortalities were observed in the control and 250 mg/kg verbenalin groups.

In the 500 mg/kg verbenalin group, one dead male was observed on Day 2, one on Day 3, and one dead female was observed on Day 2.

In the group with 1,000 mg/kg verbenalin, two dead males and three dead females were observed on Day 2 (Table 2).

Clinical signs

No abnormalities or clinical signs were identified in male or female mice of the control group during the observation period.

In the group with 250 mg/kg verbenalin dosage, soft stools and mucous stools were observed in five males and females, respectively four and six hours after dosing. And there were no other clinical signs.

In the 500 mg/kg verbenalin dosage group, soft stools, diarrhea, mucous stools, no stools and/or a decrease in fecal volume were observed in one to five males and two to five females from four hours after dosage to Day 2. Loss of locomotor activity and irregular respiration were observed in two males on Day 2. Deaths in prone position were observed in one male and one female on Day 2, and two males on Day 3.

In the 1,000 mg/kg verbenalin dosage group, soft stools, diarrhea, mucous stools and/or a decrease in fecal volume were observed

Table 2. Summary of Mortality.

Sex	Group /dose (mg/kg)	No. of animals	Day after dosing															Mortality (dead/total)
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Male	G1/0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G2/250	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G3/500	5	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	3/5
	G4/1,000	5	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2/5
Female	G1/0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G2/250	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G3/500	5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1/5
	G4/1,000	5	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	3/5

in one to five males and one to five females from two hours after dosage to Day 2. A soiled perineal region was observed in two males at four and six hours after dosing. Deaths in prone position were observed in two males and two females on Day 2, and death lying on its side resulted in one female on Day 2 (Table 3).

Body weights

During the observation period, there was no evidence of statistically significant changes in the body weights of both sexes in the group with 250 mg/kg verbenalin dosage compared with those

Table 3. Summary of Clinical Signs.

Sex	Group/dose (mg/kg)	No. of animals	Clinical sign	No. of animals affected
Male	G1/0	5	-	-
	G2/250	5	Soft stool	5
			Mucous stool	5
	G3/500	5	Soft stool	5
			Diarrhea	2
			Mucous stool	5
			Death	3
			Prone position	3
			Loss of locomotor activity	2
			Irregular respiration	2
			No stool	1
			Decrease in fecal volume	1
	G4/1,000	5	Soiled perineal region	2
			Soft stool	2
			Diarrhea	5
			Mucous stool	5
			Death	2
			Prone position	2
			Decrease in fecal volume	1
Female	G1/0	5	-	-
	G2/250	5	Soft stool	5
			Mucous stool	5
	G3/500	5	Soft stool	3
			Diarrhea	2
			Mucous stool	5
			Death	1
			Prone position	1
	G4/1,000	5	Soft stool	4
			Diarrhea	5
			Mucous stool	5
			Death	3
			Prone position	2
			Lying on side	1

in the control group.

A decrease of statistical significance in body weight was evident in males in the groups with 500 mg/kg and 1,000 mg/kg verbenalin dosage on Day 2 compared with the results in the control group (Dunnett's test, $p < 0.01$). A increase in body weight, which also demonstrated statistical significance, was exhibited in females in the group with 1,000 mg/kg verbenalin dosage (Dunnett's test, $p < 0.05$) on Day 4, and 500 mg/kg (Day 8: Dunnett's test, $p < 0.05$, Day 15: Dunnett's test, $p < 0.05$) and 1,000 mg/kg verbenalin dosage (Day 8: Dunnett's test, $p < 0.05$, Day 15: Dunnett's test, $p < 0.05$) on Days 8 and 15 compared with the control group (Table 4).

Necropsy findings

No gross abnormalities were evident in the mice (of both sexes) in any of the experimental and control groups at necropsy on Day 15 after dosing. Therefore, no additional histological or pathological analyses were conducted.

Discussion

Research and development of new medicinal herbal drugs and their extracts for the treatment of various conditions/diseases is ever-increasing. Extracts of medicinal herbs have therapeutic properties with minimal or no side effects on the human body [12]. Despite the lack of objective evidence for the safety of medicinal herbs, their prolonged use over the centuries has resulted in a false perception of safety, and widespread usage. Therefore, toxicity studies are essential in securing the safety profile of medicinal herbs which may be processed as pharmaceutical drugs [13].

The purpose of a toxicity study is to determine the lethal dose or harmfulness of a new substance. The study is divided into three phases: acute (single dose), subacute (repeated dose for four weeks), and chronic (repeated dose for three months). Among the three phases, the acute toxicity test is a short-term evaluation of the results of a single administration of a test substance. Acute toxicity studies can be used for risk assessment of chemicals to humans and organisms [14]. In addition to oral toxicity tests, various other methods are routinely conducted to comprehensively evaluate the safety of therapeutic compounds, including local toxicity tests, dependence tests, carcinogenicity tests, and reproductive toxicity tests.

Verbenalin is an iridoid glucoside, characterized by resistance to viruses, inflammation, and fungi [15], and is present in the medicinal herbs Verbena officinalis Linn [16], Cornus officinalis Siebold and Zucc [5], and other plants. Verbenalin acts as an antioxidant and has potential protective effects against cerebral ischemic damage [6,7]. Additionally, the neuroprotective property against amyloid beta-induced neurotoxicity has also been demonstrated in vitro in human neuroblastoma SH-SY5Y cells

Table 4. Mean Body Weight.

Sex	Group/dose (mg/kg)		Days after administration					Day 1-15 weight gain
			1	2	4	8	15	
Male	G1/0	Mean ± SD	29.3 ± 1.1	30.6 ± 0.7	32.4 ± 0.8	34.0 ± 0.5	35.6 ± 0.9	6.3 ± 1.5
		N	5	5	5	5	5	5
	G2/250	Mean ± SD	30.2 ± 0.8	29.1 ± 1.1	32.0 ± 0.9	34.3 ± 1.4	36.0 ± 1.4	5.8 ± 1.2
		N	5	5	5	5	5	5
	G3/500	Mean ± SD	30.0 ± 1.1	28.2 ± 0.9*	31.1 ± 1.3	32.8 ± 1.6	34.6 ± 0.9	4.7 ± 0.6
		N	5	4	2	2	2	2
	G4/1,000	Mean ± SD	30.0 ± 1.6	27.5 ± 1.0*	31.1 ± 1.8	32.8 ± 2.4	35.1 ± 2.3	5.6 ± 0.7
		N	5	3	3	3	3	3
Female	G1/0	Mean ± SD	21.8 ± 0.5	22.0 ± 0.6	23.2 ± 0.9	24.0 ± 1.3	25.3 ± 1.5	3.6 ± 1.1
		N	5	5	5	5	5	5
	G2/250	Mean ± SD	21.9 ± 0.6	20.7 ± 0.4	23.4 ± 0.6	24.2 ± 0.7	25.5 ± 0.9	3.5 ± 1.3
		N	5	5	5	5	5	5
	G3/500	Mean ± SD	22.0 ± 0.9	21.7 ± 0.5	24.2 ± 0.3	26.1 ± 0.5†	28.3 ± 1.4†	6.2 ± 1.4
		N	5	4	4	4	4	4
	G4/1,000	Mean ± SD	21.8 ± 1.3	21.1 ± 1.8	25.3 ± 1.6†	26.7 ± 1.7†	28.5 ± 0.9†	6.1 ± 0.6
		N	5	2	2	2	2	2

Significantly different from control by Dunnett's t test.
* $p < 0.01$.
† $p < 0.005$.

[4,16,17].

Although the curative effects of medicinal plants are well known these days, some are potentially toxic; therefore, toxicity studies are essential for medicinal use. For example, Lee et al [18] reported that Pinellia extracts were slightly toxic. Theoharides [19] reported human mortality in his study related to ephedrine, a component of the Ephedra herb. Jung et al [20] demonstrated that the approximate lethal dose (ALD) of Mahwangyounpae-tang-a blend of multiple herbal extracts used in Korean traditional medicine to treat respiratory diseases-was 1,000 mg/kg Mahwangyounpae-tang-a in a single oral dose acute toxicity test with ICR mice, and the target organs were the heart and kidneys.

In the case of Yukmijihwangtang, a typical Korean Medicine decoction that uses Cornus officinalis Siebold and Zucc containing verbenalin, approximately 16 g of Cornus officinalis Siebold and Zucc is contained in about 300 mL of decoction taken per day. Since Cornus officinalis Siebold and Zucc 100 g contains 322-381 mg of verbenalin [21], it can be roughly calculated that it contains 51.52-60.96 mg of verbenalin by taking Yukmijihwangtang for one day. Compared with the report that ALD was observed at 2,000 mg/kg in mice of the Yukmijihwang-tang extract [22], it was determined that verbenalin was contained in a very small amount compared with the minimum dose causing toxicity. However, in the case of verbenalin, the content of each Korean Medicine decoction varies, so it is necessary to use it safely (after several studies on safety have been conducted to establish basic data).

The test groups consisted of three verbenalin dosage groups and a control group (0.5% MC solution), with five males and five females per group. Significant results were obtained by measuring mortality, clinical symptoms, weight gain and loss, and necropsy findings for 14 days. There were no deaths in either male or female mice in the control group and the group with 250 mg/kg dosage. In contrast, four and five deaths occurred in the groups with 500 mg/kg and 1,000 mg/kg verbenalin dosage. During the observation period, soft stools, mucous stools, diarrhea, no stools, and/or a decrease in fecal volume were observed in males and/or females in the 250 mg/kg, 500 mg/kg, and/or 1,000 mg/kg verbenalin dosage groups. Loss of locomotor activity and irregular respiration were exhibited in males of the group with 500 mg/kg verbenalin dosage. A soiled perineal region was also evident in males in the group with 1,000 mg/kg verbenalin dosage. All adverse outcomes were exclusively exhibited in the animal groups exposed to verbenalin, possibly suggesting that such unfavorable effects were caused by the test substance. Furthermore, a decrease in body weight in males of the groups with 500 mg/kg and 1,000 mg/kg verbenalin dosage and an increase in body weight in females of the groups with 500 mg/kg and/or 1,000 mg/kg verbenalin dosage, all statistically significant, were notable in our study. Furthermore, a dose-dependent weight loss of statistical significance was determined in males on Day 2. These effects were accompanied by several clinical signs such as stool problems, loss of locomotor activity, irregular respiration, and prone position. It suggested that the weight loss and other adverse effects were all exclusively caused by the tested compound. However, despite the statistical significance exhibited in the increase in body weight exclusively in female mice on Days 4, 8, and 15, no clinical signs were observed. Therefore, in the case of females, a dose correlation was not recognized, indicating that these effects could not have been attributed to verbenalin.

In summary, there have been no reports of direct side effects from taking only verbenalin in other papers, and since the dose-related clinical symptoms occurred only in males, it was not possible to determine whether symptoms such as stool problems, loss of motor ability, irregular breathing, and prone position were caused by verbenalin. However, in this study, since verbenalin caused fecal and locomotor function problems in a dose-dependent manner when used in high doses, there is a possibility that fecal and locomotor function problems may occur when verbenalin is used in high doses. On the other hand, there is a possibility that side effects will not occur when a lower dose range is used.

Further experiments are needed to confirm this finding. At necropsy, no gross abnormalities were observed in any of the animals. Our findings indicated that the ALD of verbenalin was approximately 500 mg/kg in both male and female mice. Based on this ALD value, verbenalin would be classified as a low-toxicity substance (Class 3) (500-1,000 mg/kg) according to the United States Environmental Protection Agency [23]. Despite their mild to moderate severity, abnormal symptoms, such as soft stools and diarrhea, were observed in study groups with more than 500 mg/kg dosage of verbenalin; therefore, verbenalin should not be taken without the guidance of a qualified professional. Our findings, thus, establish a basis for hazard risk assessment of verbenalin by providing acute toxicity information from a single oral toxicity test. Further toxicity studies are necessary, and the management and usage of verbenalin should be exercised with caution, as toxic effects may occur beyond a certain exposure threshold.

There is a limitation in determining the level of toxicity with only a single, acute oral dose toxicity study. Therefore, additional sub-acute or chronic, repeated oral studies on toxicity and genotoxicity should be sequentially performed. Through these experiments, we believe that more accurate and scientific safety measures may be established by acquiring more information on the systematic toxicity of verbenalin. Additionally, studies are needed to determine whether there is a therapeutic or antioxidant effect at 250 mg/kg dose level where the toxicity of verbenalin has not been identified in animal/rat models.

Conclusion

We estimated the ALD of verbenalin to be 500 mg/kg in mice through the measurement of mortality, clinical symptoms, weight changes, and necropsy findings in three verbenalin dosage groups and a control group of Institute of Cancer Research mice. Although this study suggested that caution should be exercised in the use of verbenalin, other types of studies such as repeated-dose toxicity studies should be conducted for a more accurate assessment of the safety of verbenalin.

Author Contribution

Conceptualization: SHJ, LYG and HJS. Methodology: SHJ, LYG, HJS, YGS and YTH. Investigation: SHJ, LYG and HJS. Resources: LYG and HJS. Data curation: SHJ. Writing–original

draft preparation: SHJ. Writing–review and editing: SHJ and YGS. Validation: YGS and YTH. Supervision: YGS and YTH. Project administration: YTH. Funding acquisition: YTH. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All authors declare that they have no conflict of interest.

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Ethical Statement

This research did not involve any human experiments.

Data Availability

All relevant data are included in this manuscript.

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