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Research Article

Cytotoxicity and acute toxicity evaluation of hydrogen-rich *Gynostemma pentaphyllum* Makino distillate

Min Young Kim*
Toxicology Laboratory, Department of Biotechnology (Biomaterials), College of Applied Life Science, Jeju National University, Jeju, 63243, Republic of Korea

Email: jeffmkim@jejunu.ac.kr

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Abstract
Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate (HRGD) consists of *Gynostemma pentaphyllum* Makino steam distillate with hydrogen gas. Although both *G. pentaphyllum* Makino and hydrogen-rich water are well known for their biological and medical benefits, there is a lack of information on their safety and toxicity *in vivo* acute oral toxicity test and *in vitro* cytotoxicity method. The current study aimed to assess the cytotoxicity and acute oral toxicity of HRGD as a part of a safety evaluation using rat and human cell models. HRGD was administered orally once by gavage to male and female *Sprague-Dawley rats* at doses of 0, 2500, and 5000 mg/kg. Cytotoxicity assay was conducted *in vitro* at various concentrations in 10 different human normal and cancer cell lines; TK6 (human normal lymphomablastoid cells), Chang (human hepatic cells), 16HBE14o- (human bronchial epithelial cells), URotsa (human urothelium cells), MCF (human breast cancer cells), Hela (human cervical cancer cells), A375 (human malignant melanoma cells), HCT116 (human colon cancer cells), HepG2 (human liver cancer cells) and A549 (human non-small cell lung adenocarcinoma cells). From a 14-day study in rats, we observed no compound-related changes in mortality, clinical signs, body weight, food/water consumption, organ weight and gross pathology in all dose group. The result of *in vivo* acute toxicity shows that no observed adverse effect level of HRGD was below 5000 mg/kg for both sexes of rats, and the minimal lethal dose was considered to be more than 5000 mg/kg. HRGD also had no *in vitro* cytotoxicity against all tested cells. The present study data indicated that HRGD may contain bioactive compounds of potential therapeutic significance that are relatively safe from toxic effects.

Keywords: Acute toxicity, Cytotoxicity, Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate

INTRODUCTION

The global acceptance and use of herbal medicines and related products continue to assume exponential increases (Wang et al., 2021). Plant species being used, as food and medicines for nutrition and the treatment of diseases in humans and animals are reported to have serious side effects (Wyk and Prinsloo, 2020; Chand et al., 2022; Wang *et al*., 2021). Drug safety is one of the major issues in new drug development (Carr and Pirmohamed, 2020). With the development of traditional medicine, concerns about the safety and potential toxicity of herbal medicines are increasing (Jo *et al*., 2016). The general perception that herbal remedies or drugs are very safe and devoid of adverse effects is not only untrue but also misleading (Zhu *et al*., 2021; Gupta *et al*., 2021). Herbs may produce side or dangerous effects that can cause mild nausea to death. However, there is little information about the safety of herbal medicine compared with synthetic drugs (Jo *et al*., 2016; Zhu *et al*., 2021; Gupta *et al*., 2021).

Hydrogen has been noted as a novel antioxidant material. Hydrogen dissolved solutions exert antioxidant activities against hydroxyl radicals and peroxybutrites in cell-free systems and scavenge intracellular hydroxyl radicals or decrease intracellular peroxides (Hu *et al*., 2020; Sim *et al*., 2020). Some clinical trials have also determined the effect of hydrogen-rich water on several diseases, including metabolic syndrome, rheumatoid arthritis, chronic hepatitis B, Parkinson's disease and cancers (Hu *et al*., 2020; Sonf *et al*., 2022; Icjihara *et al*., 2020; Asgharzadeh *et al*., 2022).

Gynostemma pentaphyllum Makino (family Cucurbitaceae) is a perennial herb distributed mostly in Korea, Japan, China, and Southeast Asia (Nguyen *et al*., 2021). In experimental studies, *G. pentaphyllum*

Makino has been reported as a medicinal plant to have powerful biological effects such as anti-diabetics, anti-cancer, cardioprotective, hepatoprotective, neuroprotective, anti-obesity and anti-inflammatory activities (Nguyen *et al*., 2021; Wang *et al*., 2019; Liu *et al*., 2021; Yu *et al*., 2020). From phytochemical studies, it is reported thag *G. pentaphyllum* contains saponins, flavonoids, polysaccharides, amino acids and vitamins, and some essential elements (Cui *et al*., 2022; Nguyen *et al*., 2021; Weng *et al*., 2021). Chronic and subchronic toxicity of *Gynostemma pentaphyllum* with doses up to the oral dose of 750-1000 mg/kg in rats has been previously reported with no obvious toxicity and mortality (Chianthanut *et al*., 2013). However, toxicity evaluation of hydrogen-rich *G. pentaphyllum* Makino distillate (HRGD) used in that study was not performed as a prelude to future clinical studies.

In order to investigate the acute toxicity of HRGD, the acute toxicity test was performed following the guidelines and test methods for the safety tests of the drugs provided by the Food and Drug Administration, Korea. Cytotoxicity of HRGD was also evaluated to gain basic safety data for developing healthy functional food materials.

MATERIALS AND METHODS

Plant sample preparation

Gynostemma pentaphyllum (Thunb.) Makino used in this study was harvested in Jeju-do Province, South Korea. HRGD sample was provided by Youngmul company (Jeju, Korea). In brief, samples were cleaned thoroughly with water and then dried overnight. Dried *G. pentaphyllum* (Thunb.) Makino was saturated in water and then distilled by applying low-temperature vacuum extraction method. First part of the distillate was filtered to remove precipitates, filled with dissolved hydrogen gas (400 ppb) and concentrated using a rotary evaporator (Buchi Rotavapor R-200, New Castle, DE, US) and lyophilized. The yield was 16.7% based on the dry weight.

Animals

The animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals (Icjihara *et al*., 2021) and approved by Institutional Animal Care and Use Committee, Jeju National University (Jeju, Korea) for Animal ethics. Twenty-four Sprague Dawley rats of each gender were obtained from the SAMTAKO Inc. (Osan, Korea) at 6 weeks of age and used after two weeks adaptation period. Three rats per cage were housed in a room maintained at approximately 21 °C (range, 20.0–22.8 °C), relative humidity ranged from 16 to 49%, and room air was exchanged approximately 12 times per hour. The animals were allowed sterilized tap water and commercial rodent chow (Teklad Certified Irradiated Global 18 % Protein Rodent Diet, Harlan, Kansas, United States) *ad libitum* throughout the study.

Acute toxicity evaluation

Animals were randomly divided into 3 groups with 6 animals each. Each group of rats was administered singly by oral gavage with a fixed dose of the respective HRGD tested in a stepwise procedure (2500 and 5000 mg/kg body weight). The rats were fasted overnight (approximately 16 h, water was not restricted) prior to dosing. Control group rats received distilled water (vehicle) until the end of the experiment. After HRGD was administered, the rats were fasted for 4 h. The body weight of each rat was measured at the initiation of treatment and once a week during the study period. Decreased locomotor activity, salivation, soft stool, prone position, lacrimation, crouching position, convulsion, ataxic gait and incontinence of urine of the rats, and death checks were made daily for 14 days. The biopsy of dead animals during observation was performed to check the visible adverse change in internal organs.

On the 14th day, all rats were necropsied and underwent a gross examination of visible organ changes. Weights of the body and selected organs (liver, kidney, lung, spleen and heart) from the control and the test groups were measured and stored. The relative organ weight of each animal was then calculated based on absolute organ weight to body weight ratio.

Cell culture

The human normal lymphomablastoid (TK6), hepatic (Chang), bronchial epithelial (16HBE14o-) and urothelium (URotsa) cells, and human breast cancer (MCF7), cervical cancer (Hela), malignant melanoma (A375), colon cancer (HCT116), liver cancer (HepG2) and non-small cell lung adenocarcinoma (A549) cell lines were kindly gifted by Professor Wogan (Massachusetts Institute of Technology, Massachusetts, US). Chang, 16HBE14o-, URotsa, MCF7, Hela and A375 cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine with 5% CO_2 at 37 ºC. TK6, HCT116, HepG2 and A549 cell lines were grown in a medium composed of RPMI 1640, McCoy's 5A, MEM and Ham's F-12 media with supplements, respectively.

In vitro cytotoxicity

In vitro cytotoxic activity of HRGD was performed to determine cell viability by measuring the metabolism of tetrazolium substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) on ten different human cell lines in tissue culture. Briefly, cells were seeded into triplicate wells of 96-well microplate at a

density of 5×10^4 viable cells/mL. Cells were incubated with HRGD at three graded sample concentrations (200, 100 and 50 μg/mL) along with a parallel control for 72 h at 37ºC and 5 mg/ml MTT solution was added to each well. The plate was further incubated at 37°C for 4 h. Formazan crystals formed after 4 hours in each well were dissolved in 100 µL of DMSO. The plates were read immediately in a microplate reader (Dynex Technologies, Inc., Chantilly, VA, US) at 540 nm. The OD value obtained from control was taken as 100% viability. Cell viability was calculated as follows:

Experimental OD value/mean control OD value)×100 ..Eq.1

Statistical analysis

All data are presented as mean ± standard deviation of triplicate values. Significant differences between the groups were performed by using SPSS program (SPSS Inc. Chicago, IL, USA) using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Since no mortality was observed in the present study, statistical analysis for the calculation of the LD_{50} value was not performed. A *p*-value less than 0.5 was considered statistically significant.

RESULTS AND DISCUSSION

A safety report of medicinal herbs or herbal formula is a crucial step for drug development and medication counseling. Recently, the use of herbal medicines has increased; thus, the safety information is considered an important issue for the herbal prescription. The present study evaluated the safety of HRGD by *in vivo* acute toxicological analysis and *in vitro* cytotoxicity test.

Acute toxicity test

Hydrogen plays a multifaceted pharmacological role but it has yet to be widely used in medicine.

Saitoh *et al.* (2010) reported that no sub-acute toxicity was related to drinking hydrogen-rich water regarding clinical symptoms, hematology, and histopathology in rats. So far, no studies have shown that hydrogen has clear toxicity (Saitoh *et al*., 2010; Zhao *et al*., 2019).

In this study, HRGD extract was orally administrated for 2 weeks at 2500 and 5000 mg/kg/day *in vivo*. The administration of HRGD in rats did not show mortality. Moreover, changes in behavior pattern, skin color, loss of fur, or signs of illness in the rats were not observed for 14 days, thereby suggesting that HRGD is fairly non -toxic.

The administration of extracts at 2500 and 5000 mg/kg body weight to rats increased body weight over time, similar to the control group. The body weight changes serve as a sensitive indicator of the general health status of animals (Charlotte *et al*., 2021). However, all rats administered with HRGD were gaining weight. It is clearly stated that the HRGD did not inhibit the regular metabolism of rats, as confirmed by the non-significant differ-ence from control rats. These support the safety of HRGD at a given dose in rats, which resulted in an increase in body weight over time, similar to the control group, which indicated that the extracts were not *in vivo* acutely toxic (Table 1). In addition, changes in absolute organ weights were also evaluated and further, no significant differences were observed in any of the relative organ weights between the groups in either gender (Table 2).

In the present study, the LD_{50} value of HRGD by oral route was also more than 5000 mg/kg. It indicated that the HRGD could be used to further determine any preclinical and other therapeutic activities on 5000 mg/kg. However, a detailed *in vivo* subacute and chronic experimental analysis of its long-term toxicity is essential for further clinical support of HRGD. A similar trend was observed for acute toxicity of *G. pentaphyllum* extract. Numerous studies have revealed that *G. pentaphyllum* Makino exhibits numerous beneficial biological activities, making it of interest to the pharmaceutical industry (Nguyen *et al*., 2021; Gong *et al*., 2020). It has been reported that a single oral administration of *G. pentaphyllum* extract at a dose of 5000 mg/kg body weight does not produce mortality or any hazardous

Table 1. Mean body weight (g) of male and female rats receiving HRGD for 2 weeks

Group	Treatment day receiving HRGD			
	0	**1**	**7**	**14**
Male (18)				
Control (vehicle)	219.1 ± 9.04	246.5 ± 10.87	284.6 ± 11.77	329.3 ± 20.44
2500 mg/kg	220.3 ± 7.74	242.1 ± 8.69	279.7 ± 10.20	324.7 ± 14.21
5000 mg/kg	216.5 ± 8.36	241.8 ± 10.34	282.8 ± 8.73	320.0 ± 19.26
Female (18)				
Control (vehicle)	187.7 ± 8.00	207.9 ± 8.59	223.6 ± 14.78	242.7 ± 17.20
2500 mg/kg	188.2 ± 12.31	209.2 ± 7.42	221.7 ± 9.74	240.3 ± 9.26
5000 mg/kg	187.7 ± 6.50	207.5 ± 4.58	222.6 ± 8.46	242.0 ± 16.65

Values are expressed as mean ± SD of six rats

Table 2. Effect of HRGD on the relative (%) weights of organs

Organ	Control (vehicle)	2500 mg/kg	5000 mg/kg
Male (18)			
Body weight (g)	329.3 ± 20.44	324.7 ± 14.21	320.0 ± 19.26
Liver	3.3 ± 0.21	3.4 ± 0.19	3.5 ± 0.54
Kidney	0.96 ± 0.07	0.94 ± 0.12	0.91 ± 0.11
Lung	0.63 ±0.05	0.61 ± 0.06	0.64 ± 0.03
Spleen	0.25 ±0.03	0.34 ± 0.01*	0.26 ± 0.03
Heart	1.11 ± 0.06	1.12 ± 0.05	1.09 ± 0.01
Female (18)			
Body weight (g)	242.7 ± 17.20	240.3 ± 9.26	242.0 ± 16.65
Liver	3.4 ± 0.18	3.3 ± 0.43	3.3 ± 0.22
Kidney	0.89 ± 0.03	0.87 ±0.08	0.82 ± 0.07
Lung	0.59 ± 0.02	0.63± .04	0.60 ± 0.07
Spleen	0.28 ± 0.06	0.30 ±0.04	0.29 ± 0.02
Heart	0.79 ± 0.04	0.81 ± 0.02	0.85 ± 0.03*

*Mean values are significantly different at $p < 0.05$ according to ANOVA followed by Dunnett's multiple comparison test

Table 3. Cytotoxicity of HRGD in various human cell lines

Cell line	Origin	Concentration (µg/mL)			
		0	**50**	**100**	**200**
TK6	Human normal lymphoblastoid	100 ± 6.18	101.0 ± 4.08	100.1 ± 3.30	102.2 ± 4.38
Chang	Human normal hepatic	100 ± 4.88	112.4 ± 8.10	96.3 ± 8.12	101.5 ± 3.85
16HBE14o-	Human normal bronchial epithelial	100 ± 2.13	102.3 ± 7.13	98.3 ± 9.47	105 ± 4.38
URotsa	Human normal urothelial	100 ± 3.41	98.7 ± 5.17	112.2 ± 6.99	96.4 ± 3.45
MCF7	Human breast cancer	100 ± 8.87	99.8 ± 3.89	107.5 ± 5.42	106.3 ± 2.00
Hela	Human cervical cancer	100 ± 1.28	108.3 ± 5.56	103 ± 3.28	98.7 ± 1.95
A375	Human melanoma	100 ± 3.41	107.2 ± 3.12	99.1 ± 2.65	91.8 ± 11.35
HCT116	Human colon cancer	100 ± 1.18	106.5 ± 8.89	102.2 ± 6.88	102.2 ± 0.58
HepG2	Human liver cancer	100 ± 1.65	113.1 ± 5.67	116.3 ± 8.05	100.2 ± 4.75
A549	Human lung cancer	100 ± 1.77	94.3 ± 8.81	113.0 ± 5.46	99.2 ± 3.77

Values are expressed as mean ± SD (n=3)

effect *in vivo* (Chianthanut *et al*., 2013).

Cytotoxicity of HRGD

Despite the useful biological activity expressed by the plants, the study of their possible toxicity remains particularly important. Many studies have been carried out to explore the *in vitro* cytotoxic activity of the plant extract against several types of cancer. These studies have shown the anticancer properties of the extracts cancer cell lines (Ali *et al*., 2022; Mohamed *et al*., 2022).

In this study, viability of the cells was evaluated *in vitro* by MTT assay. Cells treated with various concentrations (0, 50, 100 and 200 µg/mL) of HRGD for 72 h showed that HRGD had no significant effect on the viability of cells including TK6 (human normal lymphoma-blastoid, Chang (human normal hepatic cells), 16HBE14o- (human bronchial epithelial cells) and URotsa (human urothelial cells), and MCF (human breast cancer cells), Hela (human cervical cancer cells), A375 (human malignant melanoma cells), HCT116 (human colon cancer cells), HepG2 (human liver cancer cells) and A549 (human non-small cell lung adenocarcinoma cells) up to 200 µg/mL in all tested cell lines (Table 3). Data were consistent with the results of 2 week-repeated toxicity test in the animal model (Tables 1 and 2).

Conclusion

The findings in this study suggest that the non-toxic nature of HRGD is evident from *in vivo* acute oral toxicity test when tested up to a maximum dose of 5000 mg/kg in *Sprague-Dawley rats*. During an observation period of 14 days, HRGD did not reveal any toxic signs and deaths. Also, no significant changes were observed in absolute or relative organ weights and significant adverse effects on gross pathological findings at necropsy. Furthermore, no *in vitro* cytotoxic effect of HRGD was found up to 200 µg/mL in 10 different cell lines. The current study is valuable since it could indicate that the non-toxic parts of the plant may help to employ the plant as an antioxidant and anti-inflammatory agent. Further pharmacological and chemical investigations may be required to elucidate the exact mechanism of *in vivo* and *in vitro* action of these HRGD extracts.

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Conflict of interest

The authors declare that they have no conflict of interest.

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