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

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How to Cite

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), URTosa (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 peroxybutirates 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; Sofi 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 that 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 (Chianthanat et al., 2013). However, toxicity evaluation of hydrogen-rich G. pentaphyllum 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 Youngmuk 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 (Iijihara 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 ºC), 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 urothelial (URotsa) cells, and human breast cancer (MCF7), cervical cancer (Hela), malignant melanoma (A375), colon cancer (HCT116), liver cancer (HeP2) 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% CO₂ at 37 °C. TK6, HCT116, HeP2 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 $5 \times 10^4$ viable cells/mL. Cells were incubated with HRGD at three graded sample concentrations (200, 100 and 50 $\mu g/mL$) along with a parallel control for 72 h at 37°C 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 $\mu L$ 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:

\[
\text{Experimental OD value/mean control OD value} \times 100 \quad \ldots Eq.1
\]

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 LD₉₀ 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 administered 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 difference 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 LD₉₀ 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 pre-clinical 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>
<thead>
<tr>
<th>Group</th>
<th>Treatment day receiving HRGD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Male (18)</td>
<td></td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>219.1 ± 9.04</td>
</tr>
<tr>
<td>2500 mg/kg</td>
<td>220.3 ± 7.74</td>
</tr>
<tr>
<td>5000 mg/kg</td>
<td>216.5 ± 8.36</td>
</tr>
<tr>
<td>Female (18)</td>
<td></td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>187.7 ± 8.00</td>
</tr>
<tr>
<td>2500 mg/kg</td>
<td>188.2 ± 12.31</td>
</tr>
<tr>
<td>5000 mg/kg</td>
<td>187.7 ± 6.50</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six rats
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 lymphomablastoid), 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**

Effect in vivo (Chianthanut et al., 2013).
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.

REFERENCES


