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Acute toxicity evaluation of homeopathic preparation of *Gymnema sylvestre* and analysis of its chemical constituents

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ABSTRACT

In many countries, homeopathic preparation is believed to be an effective medicine for various ailments. However, there are limited scientific evidences in regard to its usage, safety, and efficacy. It is necessary to update this age-old scientific wisdom in different aspects, including pharmacologic and therapeutic potentials. In this study, we assessed the safety profile of homeopathic preparation of *Gymnema sylvestre* (HPGS). Its chemical constituents were deciphered using LCMS approaches. HPGS was subjected to an acute toxicity study (OECD-423 guidelines) using Sprague Dawley rats. The administration of HPGS did not produce any toxic symptoms or show mortality at the dose level of 300 mg/kg body weight. Phytochemical analysis revealed that HPGS contained alkaloids, saponins, and flavonoids. These results demonstrated the non-toxic nature of HPGS *in vivo*, suggesting a long-term usage in clinical practices when administered orally.

1. INTRODUCTION

Gymnema sylvestre (Retz.) R.Br. ex Schult is a medicinal plant belonging to family Asclepiadaceae [1]. Generally, it is named as Meshashringi, Madhunashini (Sanskrit); Gur-mar, Merasingi (Hindu); Marathi: Kavali, Kalikardori, Vakundi (Marathi); Dhuleti, Mardashingi (Gujrathi); Podapatri (Telugu); Tamil: Adigam, Cherukurinja (Tamil); Sannagerasehambu (Kannada). This slow growing, perennial, and woody climber is native to Africa, Asia, Australia, and India. It has been documented in Ayurvedic system of medicine for diabetes and other disorders, such as coughing and eye pain [2].

Homeopathy is a traditional remedy based on the fundamental idea of "Similarity Principle" implying that any substances capable of causing illness in a healthy subject can be used as medicines to treat similar patterns of symptoms experienced by an ill individual [3]. There are several forms of homeopathy, including "individualized homeopathy', "clinical homeopathy," and "isopathy." In individualized homeopathy (or classical homeopathy), a single homeopathic preparation is chosen on the basis of the "total symptom picture" covering physical, mental, and emotional symptoms of a patient. The homeopathic preparation is typically manufactured through a proprietary trituration, dilution, and succussion of raw material.

Gymnema sylvestre extract has been demonstrated to show anti-diabetes activities [4]. A comprehensive review on its phytochemicals has also been conducted [5]. However, scientific evidence for its safety in homeopathic preparation is still lacking. This study aims to fulfill the knowledge gap by evaluating the safety profile of *G. sylvestre* preparation. Its chemical constituents were also deciphered.

2. MATERIALS AND METHODS

2.1. Homeopathic Preparation of *G. sylvestre* (HPGS) Preparation

HPGS was manufactured by Schwabe (GmbH, Germany) and imported by Global Homeopathic Centre (Subang Jaya, Malaysia). It was provided as a mother tincture formulation. Working HPGS was prepared freshly as an oral suspension during the dosing day. It was a mixture of 0.34 ml HPGS and 10 ml pre-

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filtered water. The preparation was adopted from the work of Surender *et al*. [6] where mother tincture were diluted with water prior to administration to rats *in vivo*.

2.2. Experimental Animals

Nine female Sprague Dawley rats were obtained from the Animal Breeding Centre, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). All rats were about 4–6 weeks old weighing between 257.23 and 291.42 g. They were kept in Makmal Bioserasi, UKM. The experiment model was approved by the UKM Animal Ethnic Committee. Each rat was housed alone in polycarbonate cages at 19°C–24°C. Food and water was given *ad libitum*. All rats were acclimatized for 5 days prior to testing.

2.3. Acute Oral Toxicity Study

Acute toxicity assessment was carried out according to OECD 423 guideline under ISO 17,025 compliance at Prima Nexus Accessory Technical Laboratory. The study was conducted on two groups of rats ($n = 3$) using a stepwise procedure. In the first step, three rats, as test group (TG), were administered orally with single dose of HPGS at 300 mg/kg body weight in 10 mg/kg vehicle (pre-filtered distilled water). Three additional rats, as control group (CG), were administered with pre-filtered distilled water only (10 ml/kg). Clinical effects on the animals were observed at 30 minutes, hourly up to 4 hours and at 6 hours of post dosing, once daily for 14 days. The rat body weight was measured on day-1, -7 and at termination (day-14). Gross necropsy was performed on both the TG and CG animals following CO_2 euthanasia at termination day. In the second step, three female rats (continuing test group, CTG) were administered orally with HPGS at 300 mg/kg body weight in 10 ml/kg vehicle following the no observable adverse effect level (NOAEL) findings from the previously TG. The rats were observed at 30 minutes, hourly up to 4 hours and at 6th hour and 8th hour post dosing for 24 hours. On day-2, the rats were sacrificed for necropsy.

2.4. Phytochemical and Macronutrient Screening

The presence of alkaloids, saponin, flavonoid, amino acid, protein, and carbohydrate in HPGS were detected based on established testing methods [7]. The qualitative results are expressed as (+) for the presence and (−) for the absence of phytochemical groups. Drangendorff test, Mayer's test, and Wagner test were used to access the presence of alkaloid qualitatively. Flavanoid and saponin were accessed by lead acetate test and foam test, respectively. Benedict's test and Molisch's test were used to access the presence of carbohydrate. Last, Million's test was used to access protein while ninhydrin test was used to access amino acid.

2.5. QTOF-LC/MS Analysis

The analysis by QTOF-LC/MS was carried out using an Agilent 6,200 series LCMS (QTOF) system (Agilent Technologies, Palo Alto, CA). The experiment was conducted at Prima Nexus Accessory Technical Laboratory. The HPLC separation was performed on a reversed-phase Zorbax SB-C18 column (250 × 4.6 mm i.d. 5 µm particle size, Agilent Technologies, Lexington, MA) at 25°C. The mobile phase consisted of water with 0.1% formic acid and 0.3% ammonia (solvent A) and methanol with 0.1% formic acid and 0.3% ammonia (solvent B) was applied with the following gradient: from 0 to 1 minute, 60% solvent B; from 1 to 2 minutes, 95% solvent B; 2–4.5 minutes, 95% solvent B; 4.5–5.5 minutes, 60% solvent B; from 5.5 to 7.0 minutes, 60% solvent B. The flow rate was 0.8 ml minutes^{-1}. Injection volume was 10 µl. The ion trap mass spectrometer was operated in positive ion mode with a scanning range from 200 to *m/z* 800. In addition, the activation energy for the MS/MS experiment was set to 1.0 V.

3. RESULTS AND DISCUSSION

3.1. Acute Oral Toxicity Test

The purpose of this study was to determine the adverse toxic effects *in vivo* following a single oral administration of HPGS. The experiment was performed on two groups of rats using a stepwise procedure. In the first step, three female rats TG were administered orally with HPGS at 300 mg/kg body weight in 10 ml/kg vehicle. Three additional female rats CG were administered with pre-filtered water only (10 ml/kg). In the second step, three female rats CTG were administered orally with of HPGS at 300 mg/kg body weight in 10 ml/kg vehicle following the NOAEL findings from the previously TG. All animals in TG, CTG, and CG survived during the course of study period without clinical or toxic manifestation. They did not show any weight loss (Fig. 1). In addition, gross lesions in the brain, kidney, lung, liver, stomach, spleen, heart, and pancreas were not detected during necropsy (Fig. 2). An animal in TG showed significant high liver and heart weight as compared to another two animals from the same TG. However, no remarkable difference was observed in the mean percentage of organ to animal body weight between the TG and CG animals. Similar findings were reported by Ogawa *et al*. [8] whereby *G. sylvestre* leaf extract was non-toxic up to 563 mg/kg/days using Wistar rat model. The exposure-related changes in body-weight, in the food consumption, in the hematological examinations, or in the serum biochemical examinations were not observed. To the authors best knowledge, this is the first scientific report on the safety profile on HPGS, and thus served as a standard to evaluate its anti-diabetic properties in the coming study. The dose applied in this study was similar with the work of on the safety profile of various mother tincture preparation. Certified homeopathic practitioner barely used mother tincture directly, and most of the practice involved small dilution of mother tincture prior to administration. Hence, 300 mg/kg dose suggest that the preparation is relatively safe when administered by common practice under the prescription of certified practitioner. Yet, further testing at the higher dose level (2,000 mg/kg) is necessary in order to classify HPGS according to OECD Guideline 423

3.2. Phytochemical and Macronutrient Screening

Plants produce a high diversity of secondary metabolites with prominent protective functions against extreme climate, predators, and harmful pathogens [9]. These secondary metabolites, termed as phytochemicals, are well-known for their medicinal values [10]. Generally, the plant secondary metabolites can be divided into three distinct groups: terpenes, phenolics, or nitrogen- and sulphur-containing compounds. In this study, classes of phytochemicals in *G. sylvestre* preparation were screened for its potential pharmacological effects. As

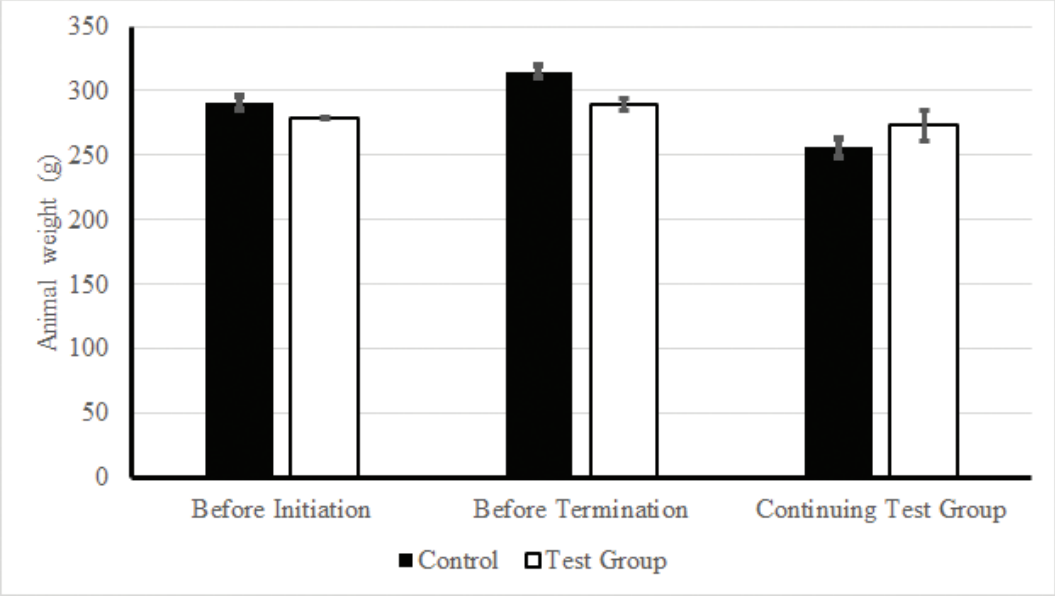

Figure 1: Weight of animal before initiation and termination of acute toxicity test.

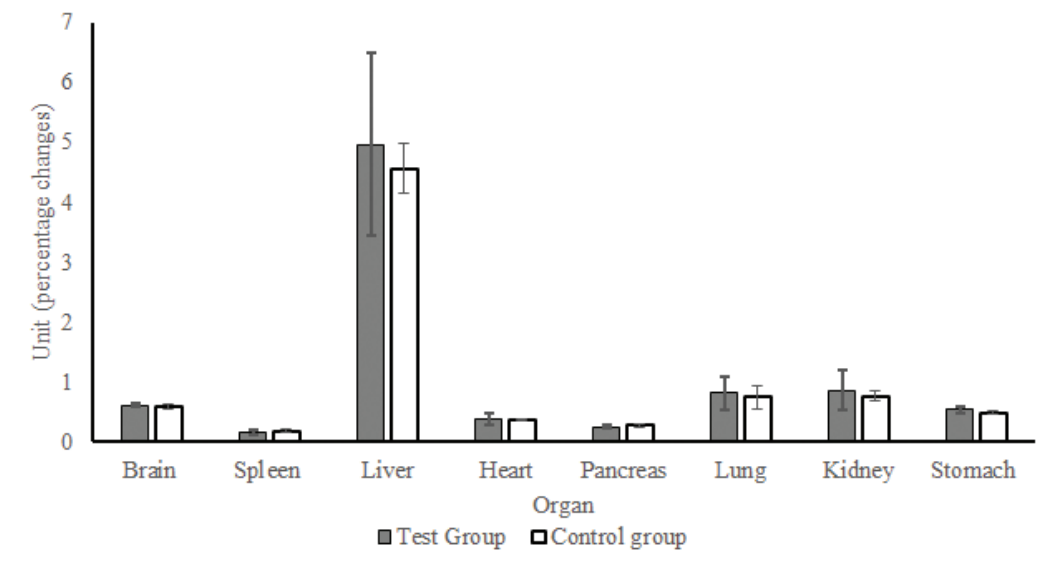

Figure 2: Comparison between test group and control group with regards of organ weight relative to animal body measured during necropsy.

tabulated in Table 1, secondary metabolites, including alkaloids, carbohydrates, flavonoids, proteins, and saponins were detected in HPGS. The finding was parallel with several reports dealing with *G. sylvestre* extract. For instance, alkaloids, cardiac glycoside, anthraquinone, tannins, phenols, saponins, and flavonoids were found in leaves part [11], whereas flavonoids, terpenoids, saponins, carbohydrates, and phenolics were found in root part [12]. Alkaloid, a nitrogen-containing compound, has been used as antiseptics, sedatives, and stomatics in Indian folk medicine [13]. Indeed, it possesses worthy pharmacological activities against inflammatory bowel disease [14] and neurodegenerative diseases [15]. There are numerous alkaloids derived from plant sources, such as atropine, berberine, sanguinarine, and quinidine [16]. On

Table 1: Phytochemical and macronutrient screening of HPGS.

Test	Observation	Inference	Phytochemicals
Drangendorff test	Dark brown color	Negative	Alkaloid
Mayers test	Light yellow color	Positive	Alkaloid
Wagners test	Brown red color	Positive	Alkaloid
Benedict test	After heat color change	Positive	Carbohydrate/sugar
Molisch test	Purple and red ring	Positive	Carbohydrate
Millons test	Brown and red ring	Positive	Proteins
Ninhydrin	Brownish green color	Negative	Amino acids
Foam test	foam present	Positive	Saponins
Lead acetate test	Yellow color	Positive	Flavonoids

the other hand, flavonoids are phenolic compounds with diverse bioactivities, such as antioxidant, anti-inflammation, anticancer, and enzyme inhibition [17]. The compounds, including quercetin, rutin, hesperidin, and myricetin, can be obtained through dietary sources. A number of saponins, especially gymnemic acids has also been isolated from *G. sylvestre* [18]. Overall, HPGS contained several natural constituents with medicinal significances.

3.3. QTOF-LC/MS Analysis

The chemical constituents present in HPGS were analyzed through QTOF-LC/MS approach. Based on standard METLIN library information (e.g., peak retention times, UV spectrum, ESI-MS/MS data), we identified 36 compounds present in the HPGS (Table 2). As compare to the findings from Chodisetti *et al*. [25] and Tiwari *et al*. [18], sterols and lipid derivatives were the common phytochemical groups found in *G. sylvestre*. In this study, [24] 5,3′,4′-trihydroxy-7-methoxy-4-phenylcoumarin 5-O-(6″-acetyl)-galactoside, [20] allamandin, [8] phytosphingosine, and [5] anacardic acid were the listed phytochemicals with pharmaceutical activities.

Table 2: Compounds identified in HPGS using QTOF-LC/MS analysis.

No.	Compounds	Reported as phytochemical	Reported with pharmaceutical activities	Reference and remark
1.	Thymine	No	No	It is a pyrimidine nucleobase.
2.	cis-Fenpropimorph	No	No	It is a morpholine-derived fungicide. Zenebe *et al*. [19]
3.	Albendazole (V)	No	Yes	It is an antihelmintic agent used predominantly in treatment of echinococcosis.
4.	tetranor-PGEM	No	No	It is a metabolite of PGE_1 or PGE_2.
5.	Cyclic de-hypoxanthine futalosine	No	No	-
6.	5,3′,4′-Trihydroxy-7-methoxy-4-phenylcoumarin 5-O-(6″-acetyl)-galactoside	Yes	Yes	Korec *et al*. [20] It is a type of neoflavonoids which exerts anti-diabetic effect *in vivo*
7.	Arg Ala Ala	No	No	It is a tripeptide.
8.	Picrotoxinin	No	Yes	Johnston [21]. It is a convulsant drug acting as $GABA_A$ receptor antagonist.
9.	Allamandin	Yes	Yes	Kuigoua *et al*. [22] It is an iridoid lactone showing antifungal, antialgal, and/or antibacterial activities.
10.	Erythronolide B	No	No	It is an intermediate of erythromycin biogenesis.
11.	9-hydroperoxy-12,13-epoxy-10-octadecenoic acid	Yes	No	It is a long-chain fatty acid.
12.	Kanamycin	No	Yes	It is an aminoglycoside bacteriocidal antibiotic.
13.	Phytosphingosine	Yes	Yes	Pavicic *et al*. [23] It is a phospholipid with anti-microbial and anti-inflammatory activities.
14.	Eicosanedioic acid	Yes	No	It is a long-chain fatty acid.
15.	±11-HEDE	Yes	No	It is a long-chain fatty acid.
16.	4,14-Dimethyl-hexadecanoic acid	Yes	No	It is a long-chain fatty acid.
17.	3-O-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoic acid	Yes	No	It is a long-chain fatty acid.
18.	Anacardic acid	Yes	Yes	Hemshekhar *et al*. [24] It has been applied for alexeritic, amebicidal, gingivitis, malaria and syphilitic ulcers in Ayurveda
19.	1-Oleoyl-2-acetyl-sn-glycerol	No	No	–
20.	(24S,25R)-25,26-epoxy-1α,24-dihydroxy-27-norvitamin D3	Yes	No	It is a lipid derivative.
21.	1α,25-dihydroxy-24a,24b,24c-trihomovitamin D3/1α, 25-dihydroxy-24a,24b,24c-trihomocholecalciferol	Yes	No	It is a lipid derivative.
22.	Neomycin B	No	No	It is an aminoglycoside bacteriocidal antibiotic.
23.	4,4-dimethyl-14alpha-hydroxymethyl-5alpha-cholesta-8-en-3beta-ol	Yes	No	It is a triterpene.
24.	2- tricosanamidoethanesulfonic	No	No	–
25.	Zymosterol intermediate 1c	No	No	–

Continued

No.	Compounds	Reported as phytochemical	Reported with pharmaceutical activities	Reference and remark
26.	MID42417:(22R)-1α,22,25—trihydroxy-26,27-dimethyl—23,23,24,24-tetradehydro- 24a,24b-dihomovitamin D3	Yes	No	It is a lipid derivative.
27.	2α-(3-Hydroxypropyl)-1α,25—dihydroxy—19—norvitamin D3	Yes	No	It is a lipid derivative.
28.	Haplophytine	No	No	–
29.	6-Deoxotyphasterol	Yes	No	It is a sterol.
30.	Ergosta-3beta,5alpha,6beta,25-tetrol	Yes	No	It is a sterol.
31.	2α-(3-Hydroxypropyl)-1α,25-dihydroxy-19-norvitamin D3	Yes	No	It is a lipid derivative.
32.	Mestanolone	No	No	–
33.	12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid	Yes	No	It is a long-chain fatty acid
34.	Sugeonyl acetate	Yes	No	Anti-inflammation
35.	MID42417:(22R)-1α,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro-24a,24b-dihomovitamin D3	Yes	No	It is a lipid derivative.
36.	Pyrimidine -2,4 (1H, 3H)-dione, 5-amino-6-nitroso	No	No	–

4. CONCLUSION

HPGS contains natural constituents, such as alkaloids, saponins, and flavonoids, without significant toxicity at 300 mg/kg consumption.

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CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

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