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Toxicologic evaluation of *Dichrostachys glomerata* extract: Subchronic study in rats and genotoxicity tests

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ABSTRACT

In western Cameroon, edible fruits and seeds from the plant *Dichrostachys glomerata* are commonly used as spices. Extract from the fruit pods has been reported as a good natural source of antioxidants and may provide health benefits. The objective of the present study was to investigate potential adverse effects, if any, of *D. glomerata* fruit pod extract (Dyglomera™) in a subchronic toxicity study and in genotoxicity studies. In the toxicity study, Sprague Dawley rats (20/sex/group) were gavaged with *D. glomerata* extract at dose levels of 0, 100, 1000 and 2500 mg/kg body weight (bw)/day for 90-days. Dyglomera™ administration did not result in mortality or show treatmentrelated changes in clinical signs of toxicity, body weights, body weight gain or feed consumption. Similarly, no toxicologically significant treatment-related changes in hematological, clinical chemistry, urine analysis parameters, and organ weights were noted. Macroscopic and microscopic examinations did not reveal treatment-related abnormalities. Mutagenic and clastogenic potentials as evaluated by Ames assay, *in vitro* and *in vivo* chromosomal aberration test and *in vivo* micronucleus test did not reveal any genotoxicity of the extract. The results of subchronic toxicity study supports the no-observed-adverse-effect level (NOAEL) for *D. glomerata* extract as 2500 mg/kg bw/day, the highest dose tested.

1. Introduction

Dichrostachys glomerata (Forssk.) also referred as *D. cinerea* and belonging to the Leguminosae family (Mimosaceae), is a deciduous small tree or shrub from Senegal to Western Cameroon, and extending across Africa to Sudan, Uganda and Zaire and also found in Asia and Australia. The plant produces edible fruits that are mostly elongated pods, fleshy, wooly, leathery or papery, containing several seeds and splitting open in the majority of cases. The pod is known as "dundu" in Hausa, "burli" in Fulani, "Kara" in Yoruba and "ami ogwu" in Igbo. The dry dehiscent constricted fruit pods of this plant are commonly used as spices in a traditional soup of the western provinces of Cameroon called "Nah po", consumed along with taro (Tchiegang and Mbougueng, 2005; Kuate et al., 2011).

In addition to its flavoring effects, the fruit and its preparations have been reported to possess several beneficial properties such as antioxidant or free radical scavenging activity (Kuate, 2010; Kuate et al., 2010; Abdou Bouba et al., 2010), anti-hypertensive effects and antibacterial effects (Fotie et al., 2004; Fankam et al., 2011). Based on the findings from *in vitro* and *in vivo* investigations, Kuate et al. (2010) reported antioxidant activity, along with LDL oxidation inhibiting property from the fruit extract of *D. glomerata*. The fruit extract has also been shown to reduce fasting serum glucose levels and lower glycosylated hemoglobin in experimental diabetic rats (Kuate, 2010). Given the growing interest and attention on the roles of polyphenols and antioxidants in human health, several investigators studied the phenolic and other chemical constituents from *D. glomerata* fruit extract prepared by using solvents such as hexane (Koudou et al., 1994), ethyl acetate (Fotie et al., 2004), and methanol (Abdou Bouba et al., 2010). In an efficacy

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; FDA, food and drug administration; GGT, gamma-glutamyl transferase; GLDH, glutamate dehydrogenase; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MNPCE, micronucleated polychromatic erythrocytes; NOAEL, no-observed-adverse-effect level; OECD, Organization for Economic Co-operation and Development; PCE, polychromatic erythrocytes.

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clinical trial with obese and obese/diabetic participants, daily administration of *D. glomerata* ground dried pods (400 mg) to normoglycemic obese groups and type-2 diabetic obese groups for 8 weeks resulted in favorable changes in body weight, BMI, waist and hip circumference, body fat, blood pressure, blood cholesterol, triglycerides, glucose, and glycosylated hemoglobin (Kuate et al., 2011). The results of this study indicate that *D. glomerata* ground dried pod may reduce cardiovascular disease risk factors in obese normoglycemic and obese type-2 diabetic human subjects.

Given the potential uses of *D. glomerata* fruit pod extract as a dietary supplement, and lack of safety-related information, adverse effects of Dyglomera™ were investigated in a long-term repeat dose toxicity study and in genotoxicity studies. In the repeat dose subchronic toxicity study, a detailed assessment of the toxic potentials of a standardized ethanolic extract of *D. glomerata* extract (Dyglomera™) prepared from dried fruits of the plant when administered daily for 90-days via oral gavage to Sprague Dawley rats was undertaken. The potential genotoxic effects of *D. glomerata* fruit extract were investigated in bacterial reverse mutation assay (Ames test) using *Salmonella typhimurium* strains, *in vitro* mammalian chromosomal aberration assay in CHO-K1 cell line, *in vivo* chromosomal aberration study in rats, and *in vivo* mammalian erythrocyte micronucleus test in rats.

2. Materials and methods

2.1. Subchronic study

2.1.1. Study design

The study was performed in accordance with (A) FDA Redbook (2000): Chapter IV.C.4.a Subchronic Toxicity Studies with Rodents, (B) Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practices (1997), (C) The standard operating procedures at Anthem Biosciences and Bioneeds and as per the mutually agreed study plan with the Sponsor, and (D) The recommendation of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for laboratory animal facility published in the gazette of India, December 15th 1998 (CPCSEA, 1998). Standard safety precautions were observed during the course of study. The study protocol (BIO-IAEC-219-10/04) was approved by the Institutional Animal Ethics Committee (IAEC) on 10th April 2010.

2.1.2. Test item

Dichrostachys glomerata fruit pod extract (Dyglomera™) used in the present study was produced by Synthite Industries Ltd., Kerala, India at a ISO certified facility that follows the HACCP protocols. The extract (Dyglomera™) is manufactured according to current good manufacturing practices (cGMP). The fruit pods from *D. glomerata* plant are collected, dried and the powder is prepared by grinding. The powder is subjected to solvent (ethanol) extraction and insoluble material is removed by filtration. The filtrate is subjected to concentration and desolvanization process under vacuum. The concentrate is checked for polyphenols and spray dried followed by pulverization and sieving to conform to 80-mesh specifications. The preparation procedure assures a consistent and high-quality product.

The extract is standardized and well-characterized. The physical characteristics and chemical specifications of the product are presented in Table 1. Dyglomera™ is a light-brown, non-fibrous powder with slightly nutty odor and taste. The powder is partly (sparingly) soluble in water. The dry powder is standardized to contain 10% polyphenols. Batch-to-batch consistency and product identification are verified by IR-analysis and HPLC fingerprint techniques. The product is routinely assayed for contaminants such as heavy metals, pesticide residues and microbial agents to ensure that the product meets the established specifications for human consumption.

2.1.3. Animals

Sprague Dawley rats (Bioneeds, Laboratory Animals and Preclinical Services, Bangalore, India), following examination by a veterinarian were used for the present study. The males and females were acclimatized for five and six days, respectively, to laboratory conditions. One hundred sixty rats were equally divided into 4 groups (20/sex/group). The average age at the time of treatment was 6–7 weeks and the average weight (±20% variation) for males ranged from 110 to 128 g, while for females it ranged from 100 to 121 g. All animals were housed under standard laboratory conditions and in accordance with CPCSEA (1998) guidelines. The animals were fed with Nutrilab rodent feed (Provimi Animal Nutrition India Pvt. Ltd., Bangalore, India) throughout the acclimatization and experimental period. All animals were provided with clean and potable drinking water in polypropylene water bottles with stainless steel sipper tubes.

2.1.4. Treatment

The rats divided into four groups were treated orally (gavage) once daily with *D. glomerata* fruit pod extract (Dyglomera™) preparation at dose levels of 0 (Group I – vehicle control; distilled water), 100 (Group II – low dose), 1000 (Group III – mid dose), and 2500 (Group IV – high dose) mg/kg bw (dose volume 10 mL/kg) for 90 consecutive days. The available historical use of *D. glomerata* pod as an edible fruit spice and also human clinical study indicates innocuous nature of the extract. The dose selection for the study was based on these observations, intended maximum consumption in humans and the preliminary dose range finding study of Dyglomera™ in rats which was conducted prior to the main study. The 14-day dose-range findings study at dose levels 0, 100, 1000 and 2500 mg/kg bw/day revealed no adverse toxic effects in rats. Based on these observations, it was concluded that the test item *Dichrostachys glomerata* extract administered to Sprague Dawley Rats by oral gavage was non-toxic at doses up to 2500 mg/kg/day under the experimental conditions and doses employed. Based on pre-formulation assessment, distilled water was found to be a better vehicle on physical observation as compared to the other suspending agents. For both 14-day and 90-day toxicity study, formulation in distilled water was prepared freshly on a daily basis and was vortexed before administration to each rat. The test item was stable in water even after 24 h.

2.1.5. Parameters investigated

2.1.5.1. Clinical examination, body weight and feed consumption. All animals were subjected to detailed clinical examinations before initiation of the treatment and weekly thereafter (varied by ±1–2 days) during the study. Individual animal body weights were recorded at receipt, on the day of initiation of treatment and weekly thereafter (±2 days) during the course of study. Fasting body weights were recorded at terminal euthanasia. Individual animal feed consumption was recorded weekly (±2 days) except for the animals during interim blood collection. Group mean feed consumption was calculated.

2.1.5.2. Neurological/functional, ophthalmoscopic examination. Neurological/functional examination was carried out during 12th week for control and high dose group animals. For neurological examinations cage-side observations included movements (gait), respiratory pattern, skin and hair coat, salivation, and lacrimation. Neurological observations on examination table included tactile response and response to tail pinch-unreceptive stimulus. Additional neurological measurements included locomotor activity, head shaking, grasping strength and equilibrium test. Functional observation record included visual response, auditory response, and response to proprioceptive stimulus (gait, landing foot splay, righting reflex). Ophthalmological examination was performed on all animals before the study begins and on control and high dose group animals during 12th week of the study.

2.1.6. Clinical pathology

2.1.6.1. Hematology. Blood samples were collected from 10 rats of each sex per group on Days 15, 45, and at termination (day 91). The animals were fasted overnight before blood collection. Water was provided *ad libitum* during fasting period. Blood samples were collected from the animals separately into tubes containing K_2-EDTA and heparin for hematology and clinical chemistry, respectively. A series of hematology parameters as listed in Tables 2 and 3 were analyzed using Sysmex, KX-21, Hematology analyzer (Transasia Bio-Medicals Ltd., India). Standard microscopy of blood smear, stained with Leishman's stain, counting 100 cells was performed to count neutrophils, lymphocytes, eosinophils and monocytes.

2.1.6.2. Clinical chemistry. Plasma was separated by centrifuging the blood samples at 5000 rpm for 10 min and a series of clinical chemistry parameters listed in Tables 4 and 5 were analyzed using the "EM-360 Fully automated clinical chemistry analyzer" (Transasia Bio-Medicals Ltd., India). Sodium and potassium was estimated using Easylyte Na/K analyzer (Medica Corporation, USA).

2.1.6.3. Urinalysis. On completion of 90-days of treatment period, urine was collected in metabolic cages from 10 rats of each sex of the group. Animals were fasted overnight but water was provided *ad libitum* during this period. The urine was analyzed for the following parameters: appearance, color, volume (for 12 h), pH, specific gravity, occult blood, leucocytes, bilirubin, urobilinogen, ketone bodies, proteins, glucose, nitrite and microscopic examination of urine sediments.

2.1.7. Pathology

2.1.7.1. Gross necropsy. At the end of treatment period (day 90), all the animals were fasted overnight. The next day, animals were weighed before exsanguinations and euthanized using carbon dioxide asphyxiation. The external and internal gross pathological examination was conducted.