In Vivo Genotoxicity Testing of *Sesbania grandiflora* (Katuray) Flower Methanol Extract

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Abstract—**The booming interest in using natural compounds as an alternative to conventional medications has paved way to focus the attention on plants that provide rich sources of bioactive phytochemicals. For regulatory purposes, evaluation of the genotoxic and cytotoxic effects of such alternatives is therefore empirical as part of the plant's hazard assessment. *Sesbania grandiflora* is among the plants used as a traditional remedy in folk medicine and a subject of research for its medicinal benefits. This study aimed to evaluate the genotoxic potential induced by *S. grandiflora* Flower Methanol Extract (SGFME) in terms of the frequency of Micronucleus Polychromatic Erythrocyte (MNPCE) and PCE ratio employing the micronucleus assay. The frequency of MN was examined in Bone Marrow Cells (BMCs) obtained from male Swiss albino mice exposed in vivo to four different concentrations (11.25, 22.5, 40, and 90 mg/kg) of SGFME and MMC (70 mg/kg; positive control) and sacrificed 24 h post-intraperitoneal injection. Results showed a significant ($p < 0.01$) rate of MNPCEs for 11.25 and 22.5 tested concentrations of SGFME and is comparable with the MMC-treated mice. Although PCE ratio values in all doses of SGFME-treated mice were over 0.20, it is worth noting that 40 and 90 tested concentrations of SGFME-treated mice exhibited the lowest value, i.e., 0.22 and 0.28, respectively. The present study has demonstrated that *S. grandiflora* possesses genotoxic potential for murine BMCs. This activity could be ascribed from the bioactive compounds present in *S. grandiflora* that require further isolation and characterization of the active molecules.**

Index Terms—**genotoxicity, micronucleus, phytochemicals, *Sesbania grandiflora***

I. Introduction

In recent times, there has been an upsurge of interest in the use of traditional medicines, otherwise known as ethnobotanicals (EBs) or phytomedicines, within community health care systems or in communities for both developing and developed countries. This growing interest, however, have also raised considerations of its need for regulation to ensure the safety, efficacy, and quality of EBs. Before the widespread use of manufactured drugs, EBs are important resource in the promotion of health and has contributed significantly in striving to fight a vast number of diseases. It is also essential to point out that most drugs were developed from medicinal plants, and that culture and history has greatly influenced the use of EBs in modern societies. Despite the promising potential of a number of EBs, many of them remain untested and their use are either poorly monitored or not monitored at all. The outcome of this is a poor understanding of their mechanism of action, potential side effects, contraindications, and food and drug interactions to promote the safe and rational use of these agents. It is therefore imperative to guarantee that all EBs are safe and of suitable quality.

The Philippines, being rich with natural resources, has plenty of plants that are noted for their medicinal values and many of which have been studied for their efficacy by means of isolating their active constituents and evaluating their pharmacological actions. There are, however, still a number more of these plants that are used extensively in folk medicine that needs further investigation, one of which is *Sesbania grandiflora.* A native to Asia and tropical regions on low and medium altitudes, *S. grandiflora L.* Pers., locally known as *katuray*, is a perennial, deciduous tree which can grow to a height of about 10 – 15 m with a life span of around 20 years [1]. All of its parts have been used as a traditional remedy in folk medicine to treat various diseases. Despite the emphasis on the medicinal values of *S. grandiflora*, few focused on the adverse implications. A member of the family *Fabaceae, S.grandiflora* shares the same secondary metabolites—alkaloids, glycosides, and phenolics—as the rest [2], [3]. Because a number of secondary metabolites have incongruent distribution pattern among its members, this may justify the genotoxic capacity of *S. grandiflora*.

In this study, we investigated the genotoxic potential of *S. grandiflora* using the micronucleus test. The assay evaluates chromosomal instability in terms of the number of micronuclei polychromatic erythrocytes (MNPCEs) and PCE ratio (PCE/(PCE + normochromatic erythrocytes (NCE)) ratio.

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II. Methodology

A. Test Animals and Husbandry

Thirty male Swiss albino mice (2 – 2.5 months old) weighing 20 – 30 grams were used in the study. Prior to the start of the study, test subjects were subjected to a 5-day acclimatization period in a temperature (30°C) and humidity (30-35%) controlled room with a light:dark cycle of 12h:12h. Test animals were allocated an improvised polycarbonate cage. Standard mice feeds and water were supplied *ad libitum*. Animals were weighed daily.

B. Ethical Clearance for Animal Experimentation

The study was conducted in accordance with the care and use of laboratory animal guideline approved by the Institutional Animal Care and Use Committee (IACUC) of Virgen Milagrosa University Foundation and Department of Agriculture – Bureau of Animal Industry (DA-BAI).

C. Extraction of Plant Material

Flowers of *S. grandiflora* var. *grandiflora* were air dried for 7 days. Dried flowers were then cut and soaked in methanol (100 g in 1L of solvent) for 7 days and extracts were later on filtered. The supernatant was subjected to rotatory evaporation and were kept in sterile universal bottles and stored in refrigerator at -20˚C until further use.

D. Grouping and Dosing

The animals were divided into six groups, each having five mice. In the preliminary research done by the Department of Science and Technology – National Academy of Science and Technology (DOST-NAST), they tried to obtain the LD_{50} of intraperitoneally-injected SGFME in white mice and found no acute toxicity even at 112.5 mg/kg [4]. Following the result in the study by DOST-NAST, concentrations of SGFME employed in the study were 11.25 ($SGFME^1$), 22.5 ($SGFME^2$), 45 ($SGFME^3$), and 90 ($SGFME^4$) mg/kg. Distilled water was used as a vehicle for the delivery of extracts. Dosing of control negative (distilled water) and control positive (mitomycin C [MMC]; 70 mg/kg single dose) was also included. Extracts and negative control were administered intraperitoneally in a single dose. All abnormal clinical signs were recorded before and after dosing.

E. Bone Marrow and Microscopic Slide Preparation

After the 24 h administration of extracts and controls, bone marrow were isolated from the femur in 1 ml of Fetal Bovine Serum (FBS), subjected to centrifugation at 3000 rpm for five minutes, and smeared on slides. Supernatant fractions were decanted and sedimented cells were collected. Smears of the bone marrow were dried and fixed within 10 – 20 min immersion in methanol. Bone marrow slides were stained with May-Grünwald-Giemsa stain and randomly coded.

F. Observation and Recording of Micronuclei

Bone marrow slides were sent to Region 1 Medical Center (Dagupan City, Philippines) for observation and reading under an electric microscope by two resident pathologists. Criteria for scoring micronuclei were based upon the scoring set forth by MEDETOX [5]. Results were expressed as the number of MNPCEs in 2000 PCEs. Mean number of MNPCE ± standard deviation was calculated for each treatment group. Additionally, the PCE ratio (PCE/(PCE + normochromatic erythrocytes (NCE)) ratio was also calculated by counting 1000 erythrocytes, for detecting the possibility of cytotoxicity [6].

G. Statistical Analysis

All variables follow non-normal distribution, hence, data transformation was conducted using two-step transformation through fractional rank case then inverse distribution function. Three variables were transformed, i.e., PCE, NCE, PCE Ratio, and were subjected to one-way ANOVA followed by LSD post hoc analysis due to inconsistencies of Tukey's post hoc. Result of statistical evaluation was regarded as significant at $p < 0.05$. In addition, a PCE ratio of > 0.20 was considered acceptable [6]. Statistical analyses were carried out using SPSS for Windows.

III. Results and Discussion

A. Clinical Signs of Animal Toxicity

Evident signs of acute toxicity a few minutes after the injection of SGFME include laboured breathing, reddening and fanning of the ears, writhing, and hind leg paralysis. Convulsions and death then followed after 7 minutes to 1 hour. Acute toxic effects of *S. grandiflora* may be attributed to the presence of histamine-like compound or the liberation of histamine in the body after ingestion of the plant's flowers and pods as affirmed by Fojas *et al.* [4] in a drug-antagonism screening test of *S. grandiflora* on the autonomic nervous system done on guinea pig ileum. They showed that the ileum was stimulated by katuray extracts and such stimulation was inhibited by mepyramine, an anti-histamine drug. Histamine, a biogenic amine, plays important biologic roles in living cells; however, in larger amounts it may pose toxicity [7]. When subcutaneously injected, histamine causes gastric acid secretion in the stomach, bronchiole constriction leading to dyspnea, and capillary dilatation leading to flushing of the skin [6], [8]. Topical application of histamine produces a so-called triple response: (a) flushing/reddening of the skin, (b) urticarial wheal due to local edema, and (c) a surrounding arteriolar flare due to an axon reflex. Intravenous injection of histamine leads to lowering of blood pressure by acting on smooth muscles of blood vessels and causing profound hypotension and capillary dilatation. Thus, the gasping and faster respiratory rate are most likely due to bronchiole constriction; reddening and fanning of the ears as a consequence of capillary dilatation; and writhing possibly as a result of the gastric acid secretion in the stomach. Convulsions and death after 5 to 20 minutes in histamine-treated guinea pigs were also observed and autopsy revealed that the lungs were fully inflated and

remained so even after its removal [9], thus suggesting complete bronchoconstriction.

Fojas *et al.* [4] and Kamei *et al.* [10] further noted that after the extract's first initial reaction to white mice, i.e., stimulation, it is then followed by CNS depression and is manifested by the loss of pinna reflex, righting reflex and muscle coordination, and body flaccidity. Among these CNS depression signs, body flaccidity as manifested by hind leg paralysis was observed. Additionally, histamine-induced mortality in hypersensitive mice is due primarily to the extravasation of blood volume into the extravascular space leading to circulatory collapse for which the animal is unable to compensate [11].

B. *Genotoxicity of Sesbania Grandiflora Flower Methanol Extract*

Fig. 1A, 1B, and 1C summarizes the MNPCE, PCE, and NCE frequencies, respectively in bone marrow cells (BMCs) of mice treated with SGFME and MMC (positive control). All tested concentrations of SGFME were found to induce chromosomal damage, i.e. genotoxicity, as indicated by the presence of MNPCEs. However, LSD post hoc analysis revealed that MMC is comparable with SGFME^{1} and SGFME^{2}, but significantly different to SGFME^{3} and SGFME^{4}. There are significantly more MNPCEs in MMC, SGFME^{1}, and SGFME^{2} treatments as compared to the observed frequency of MNPCEs in SGFME^{3} and SGFME^{4} [(MMC = SGFME^{1} = SGFME^{2}) > (SGFME^{3} = SGFME^{3})]. PCE ratio > 0.2 is considered acceptable, i.e., no prevailing bone marrow cytotoxicity. Result of this study showed that all SGFME concentrations including MMC do not induce bone marrow cytotoxicity (Fig. 1D). It is important to note however, that SGFME^{3} and SGFME^{4} are slightly near the PCE ratio border, indicating that doses 40 and 90 mg/kg are close to causing bone marrow cytotoxicity. Our data suggest that the decrease of PCE ratio was attributable to an increase in the frequency of NCE (Fig. 1C) which may be suggestive of inhibition of cell division and maturation, killing of erythroblasts, elimination of damaged cells, or dilution of existing pool of cells with newly formed ones [12]. Fig. 2 presents a pictograph of bone marrow smear with an MNPCE.

Genotoxicity is the ability of an agent to induce damage to DNA and cellular components involved in the functionality and behavior of chromosomes within the cell [13]. Damaged DNA in human cells, when detected, would initiate a DNA damage response, leading to its repair; hence, avoiding its transmittance to daughter cells [14]. Despite such response, changes in the genome are inevitable, and cells, i.e. proliferating cancer cells, becomes prone to genomic instability [14].

Bone marrow erythroblast customarily develops into PCEs with the main nucleus expelled. PCEs are young erythrocytes that contain mostly RNA [15], and as it further matures, it loses its RNA and contains primarily hemoglobin, now becoming an NCE. NCEs mature red blood cells however are smaller than PCEs. In damaged-induced cells however, a micronucleus remains within the enucleated cytoplasm of the said cell [16]. Micronuclei (MN), also known as Howell-Jolly bodies, are extra-nuclear chromatin bodies of structural or numerical chromosomal aberrations separated from the main nucleus following cell division. MN are generated defects of induced genotoxic agents exhibiting clastogenic and aneugenic effects on cell division and cell cycle [17]. As increase in the number of MNPCEs is indicative of induced chromosomal damage they may be used to quantify the genotoxicity of chemical and physical compounds [18]. Cytotoxicity index, on the other hand, may be obtained in the micronucleus assay by getting the PCE to NCE ratio between the test-agent treated animals and vehicle-control. The underlying mechanism behind MN as products of clastogenic attacks are unrepaired Double-Strand Breaks (DSBs) or by misrepair of various DNA lesions [19]. These misrepaired or unrepaired DSBs may well explain the occurrence of clastogen-induced MN on target cells. In addition, MN may also result from exposure to aneugens. Activity that generates daughter cells with abnormal chromosome numbers demonstrates aneugenicity. The spindle inhibitory activity of aneugens is what makes it the most common form of genomic instability in cancer cells [20].

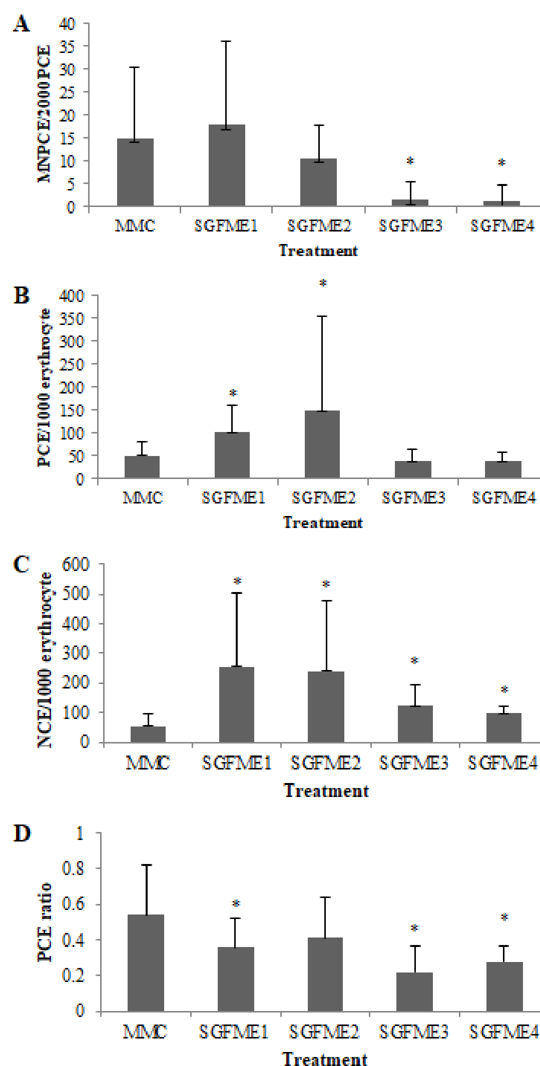

Figure 1. MNPCE (A), PCE (B), and NCE (C) frequencies and PCE ratio of bone marrow cells of male Swiss albino mice 24 h after treatment with *Sesbania grandiflora* flower methanol extract (SGFME) and mitomycin-C (MMC).

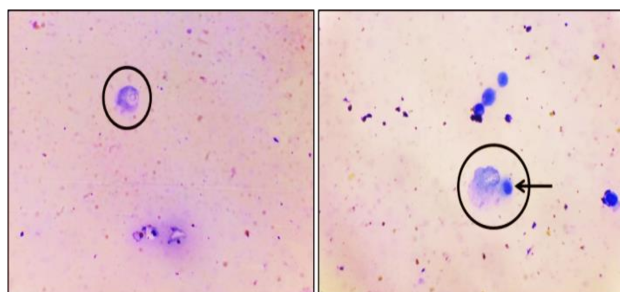

Figure 2. A pictograph of Swiss albino mice whole bone marrow smear showing enucleated PCE (circle) containing micronuclei (arrow).

Despite the many beneficial phytochemicals found in *S. grandiflora*, it is also significant to take into account the possibility of it having phytotoxins, in addition to its possible adverse reactions with prescription drugs. Phytotoxins serve as a form of defense against potential enemies, including insects, pathogenic microorganisms, and herbivorous animals. Additionally, it promotes plant survival against abiotic factors. Among the most frequently encountered phytotoxins include alkaloids, cyanogenic glycosides, glucosinolates, isothiocyanates, and furanocoumarins [21]. *S. grandiflora* belongs to the Family *Fabaceae* which are known to produce diverse secondary metabolites, including toxic ones such as pyrrolizidine alkaloids [22], rotenoids (rotenone) [23], and quercitin [24], [25].

Pyrrolizidine Alkaloids (PAs) are strong clastogenic agents that can produce MN in hepatocytes, BMCs, and peripheral blood cells. Administration of monocrotaline found in the seeds of *Crotalaria* for a period of 6 days increased the frequency of MNPCEs in peripheral blood samples [26]. Riddelliine in Chinese Hamster Ovary (CHO) cells [27], heliotrine, lasiocarpine, petasitenine and senkirkine in V79 cells [28], and integerrimine in C57Bl/6 mice BMCs [16], when appropriately metabolically activated, induce chromosomal aberrations. PAs are also inducers of Sister Chromatid Exchange (SCE) [27], [29] which is a product of DNA homologous recombination in response to collapse of the replication fork as a result of DNA strand breaks. Such SCEs has been associated with chromosome damage and tumor induction. Unscheduled DNA syntheses (UDS) are also notable with regards to PA exposure indicating DNA damage [30].

Rotenoids in the form of rotenone in the acetone extract of *Pachyrhizus erosus* (yam bean; *Fabaceae*), tropical herb, native from Mexico and Central America, demonstrated cytotoxic and genotoxic activity against K562 human leukemia cell lines [23]. Genotoxic capacity of rotenone was detected thru induced cell death, and caspase-3 activation as indicated in their assessment thru TUNEL assay, immunocytofluorescence, and comet assay. Experimental studies in mouse L5178Y lymphoma cells also resulted in a positive genotoxic result [31]. Rotenone is considered to be a spindle poison, and that numerical aberrations (hypodiploidy, hyperdiploidy, polyploidy, endoreduplication) in cultures of Chinese Hamster Ovary (CHO) cells [32] and micronuclei in cultures of human lymphocytes [33] are evident, but not structural chromosomal aberrations. The increase in the incidence of micronuclei in CHO cells was said to be in a concentration-dependent manner. Guadaño *et al.* [34] elaborated that the presence of MN without any chromosomal aberrations suggests that rotenone disturbs the distribution of mitotic chromosome.

Quercitin is a flavonoid derivative that occurs in edible plants mainly in the form of glycosides, such as isoquercetin and rutin. Quercitin was found to induce micronucleus formation in human lymphocytes [35]. Mouse and hamster quercitin-exposed cells were also detected to have mutations at the *tk* locus of human lymphoblastoid cells [36]. SCE and MN were also notable in Chinese hamster cell lines including CHO [37], V79 [36], and mouse erythrocytes [26].

IV. Conclusion and Recommendations

In the present study, all SGFME-treated mice showed detrimental effects that arose from a single dose of the extract within a 24 h period most likely due to the presence of histamine. Furthermore, inductions of MNPCE were detected with all doses of SGFME. These data would confirm the chromosomal damage exerted by SGFME owing to pyrrazolidine alkaloids, rotenone, and quercitin; although no bone marrow toxicity has been observed. Nevertheless, further mutagenic studies that measure the different levels of DNA damage, i.e. recognition of the centromere and kinetochore sites in formed MN, are imperative and may further increase the validity of the study.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Levylee G. Bautista, Dawn Grace E. Santos, and Ghafoor A. Haque, Jr. I devised the project, the main conceptual ideas, and proof outline. Levylee G. Bautista, Dawn Grace E. Santos, Aishwarya V. Veluchamy, Jesusa E. Santos, and Ghafoor A. Haque, Jr. I carried out the experiments. Levylee G. Bautista, with the help of a statistician, performed the analytical calculations and numerical simulations. Levylee G. Bautista and Dawn Grace E. Santos took the lead in writing the manuscript. Rodolfo T. Rafael supervised the findings of this work. All authors provided critical feedback and helped shape the research and manuscript.

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