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GENOTOXICITY EVALUATION OF POLYPHENOLIC BIOINSECTICIDE FROM STREBLUS ASPER (PBSA) USING MOUSE BONE MARROW MICRONUCLEUS ASSAY

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ABSTRACT: Isolation of plant-based pesticides gaining recent interest due to the clastogenic implications of synthetic insecticides. The polyphenolic-rich fraction from the stem bark of *Streblus asper* was proven as an insecticide against *Dysdercus cingulatus*. This study aims to evaluate the genotoxicity of polyphenolic bioinsecticide from *Streblus asper* (PBSA) using the mouse bone marrow micronucleus assay and compare its effect with malathion, an organophosphorus insecticide, and vepacide, a neem-based bioinsecticide. The micronucleus assay was conducted after 24 and 48 hours after the second administration of the pesticides (two doses by i.p. injection for two consecutive days). Swiss albino mice were divided into six groups, each comprising six animals. The first group received dimethyl sulphoxide (Group I - DMSO control), the second group (Group II - positive control) received 100mg cyclophosphamide/ Kg body weight, Group III received 276mg (LD_{10}) malathion/ Kg body weight and Group IV received 1000mg (LD_{10}) vepacide/ Kg body weight. Group V and VI received 500 and 1000mg polyphenolic-rich fraction (PBSA)/ Kg body weight respectively. A significantly higher frequency of micronuclei was observed in malathion and vepacide administered animals when compared to the DMSO control group whereas in the case of PBSA, no significant micronuclei formation was observed. The study concluded that polyphenolic rich fraction (PBSA) fails to influence the induction of micronuclei by proving that it has no cytogenetic toxic potential.

INTRODUCTION: The micronucleus assay using immature bone marrow erythrocytes of mice has been widely used as a simple and sensitive short-term screening method *in-vivo* for determining the mutagenicity of chemical substances [1–3]. As this assay uses "whole animals", it has the merits of including such factors as absorption, distribution, and metabolism of the chemical substances in the evaluation [4].

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Synthetic pesticides are considered as a potential mutagen in mammalian *in-vivo* systems. During the co-evolution of plants and insects, plants have biosynthesized several secondary metabolites to serve as defense chemicals against insect attacks. These bioactive chemicals may serve as insecticides, antifeedants, growth inhibitors, repellents, *etc*.

Scientists now turn to the isolation of eco-friendly plant-based pesticides due to the clastogenic implications of synthetic pesticides. Several polyphenolic compounds have been already reported to have insecticidal action [5, 6, 7]. *Streblus asper*, Lour. (Family- Urticaceae, Subfamily- Moraceae) is a traditionally used well-known

medicinal plant in India. In Ayurveda, stem bark from the *S. asper* plant is prescribed for the treatment of elephantiasis, for which there is currently no proven cure in modern medicine. Folklore medicine also claims that stem bark can heal diseases like cancer, ulcers, diarrhoea, dysentery, toothaches, *etc* [8]. The stem bark is reported to be effective against lymphoderma, chyluria, and other manifestations of filariasis [9, 10] and is useful in foul ulcers, diarrhea, dysentery, inflammations and fever [11]. Various extracts of *Streblus asper* have been shown to possess antiparasitic and antimicrobial action [12, 13]. The polyphenolic-rich fractions from the stem bark of *Streblus asper* exhibited promising insecticidal activity against *Dysdercus cingulatus* [5]. The main objective of this study was to find out whether polyphenolic fraction from *Streblus asper* (PBSA) causes any genotoxic effects at the chromosome level as evidenced by the micronucleus (MN) formation in mouse bone marrow cells.

MATERIALS AND METHODS:

Chemicals: All chemicals were purchased from Sigma St. Louis, MO, USA. Solvents were purchased from Merck, India. Vepacide (Azadirachtin 12%) purchased from the local market and malathion (technical grade, 98% pure) obtained from Premier Pesticide Ltd., India were used for the study.

Bioinsecticide from *Streblus asper*: The stem bark of the plant, *Streblus asper* was collected from Nagarcoil Forest (Tamilnadu, India) and was authentically identified by Dr. Valsaladevi, Curator, Department of Botany, University of Kerala. The specimen was deposited in the herbarium of the Department of Botany, University of Kerala (Voucher No: KUBH 5702). Polyphenolic compounds were extracted from *Streblus asper* according to the procedure reported earlier from this laboratory [5]. Chloroform [fraction C] obtained from silica gel chromatography was subjected to silica gel thin layer chromatography using 30% acetic acid as a solvent system and dried plates were illuminated under UV light. Two blue spots [spot I with an Rf of 0.482 (compound I) & spot II with an Rf = 0.589 (compound II)] were obtained and both were eluted in chloroform, the solvent was evaporated in vacuum, and the compounds were red is solved in 40% ethanol and used for testing the insecticidal action on *D. cingulatus*. The maximum insecticidal activity was shown by compound I (spot I: Rf - 0.482) with an LD_{50} of 0.894 µg/insect by residual film technique and 0.595 µg/insect by topical application [14]. This most active polyphenolic bioinsecticide from *Streblus asper* (PBSA) was used for its mutagenicity evaluation by mouse bone marrow micronucleus test.

Animals: Swiss albino mice (body weight ranging between 16 and 20 g) were obtained from Animal House, Department of Biochemistry, University of Kerala, Thiruvananthapuram, India. Animals were housed in standard polypropylene cages which were maintained under standard conditions of temperature and humidity and were supplied food (M/S Lipton India Ltd., Calcutta, India) and water *ad libitum.* Animals were handled by the laboratory animal welfare guidelines [15]. Animal experimentation was conducted by the institutional ethical guidelines for the conduct of the experiments on laboratory animals as per CPCSEA rules (Sanction No: IAEC-KU18/05-06-BC-KSD).

Methodology: The micronucleus assay was conducted 24 and 48 hours after the administration of the samples. Animals were divided into six groups and each group consisted of six animals. Group I, which served as control, received only 0.1 ml DMSO, group II, which served as a positive control, received 100mg cyclophosphamide [CP] / Kg body weight, group III, received 276mg (LD_{10}) malathion/ Kg body weight and group IV received 1000mg (LD_{10}) vepacide/ Kg body weight. Group V and VI received polyphenolic rich fraction of *Streblus asper* (PBSA) at dose levels of 500 and 1000mg/ Kg body weight respectively **Table 1**. Insecticides dissolved in 0.2 ml 10% DMSO were administered intraperitoneally for two consecutive days and all the animals were killed by euthanasia after 24 and 48 hours. Both femora were removed through the pelvic bone just below the knee and the bone marrow was flushed into a tube containing 3 ml fetal calf serum. The tubes were then centrifuged at 1000 rpm for 10 minutes. From the pellet, smears were made on slides, and the air-dried preparations were stained by the May-Grunwald –Giemsa method [16]. For each mouse, three slides were prepared. Evidence of micronuclei in polychromatic (P) and

normochromatic (N) erythrocytes was observed in a light microscope at 1000 × magnification. 2000 polychromatic erythrocytes were evaluated per animal; simultaneously, the number of normochromatic erythrocytes was also scored [17, 18] **Fig. 1.**

TABLE 1: TREATMENT PROTOCOL AND EXPERIMENTAL DESIGN

Sl. no.	Groups	Treatments	Dose
1	Group I	DMSO	0.2 ml 10 % DMSO
2	Group II	(+) Control	100mg cyclophosphamide [CP] / Kg body weight dissolved in 0.2 ml 10% DMSO
3	Group III	Malathion	276mg (LD_{10})/ Kg body weight dissolved in 0.2 ml 10% DMSO
4	Group IV	Vepacide	1000mg (LD_{10})/ Kg body weight dissolved in 0.2 ml 10% DMSO
5	Group V	PBSA 1	500 mg/ Kg body weight dissolved in 0.1 ml DMSO
6	Group VI	PBSA 2	1000mg/ Kg body weight dissolved in 0.2 ml 10% DMSO

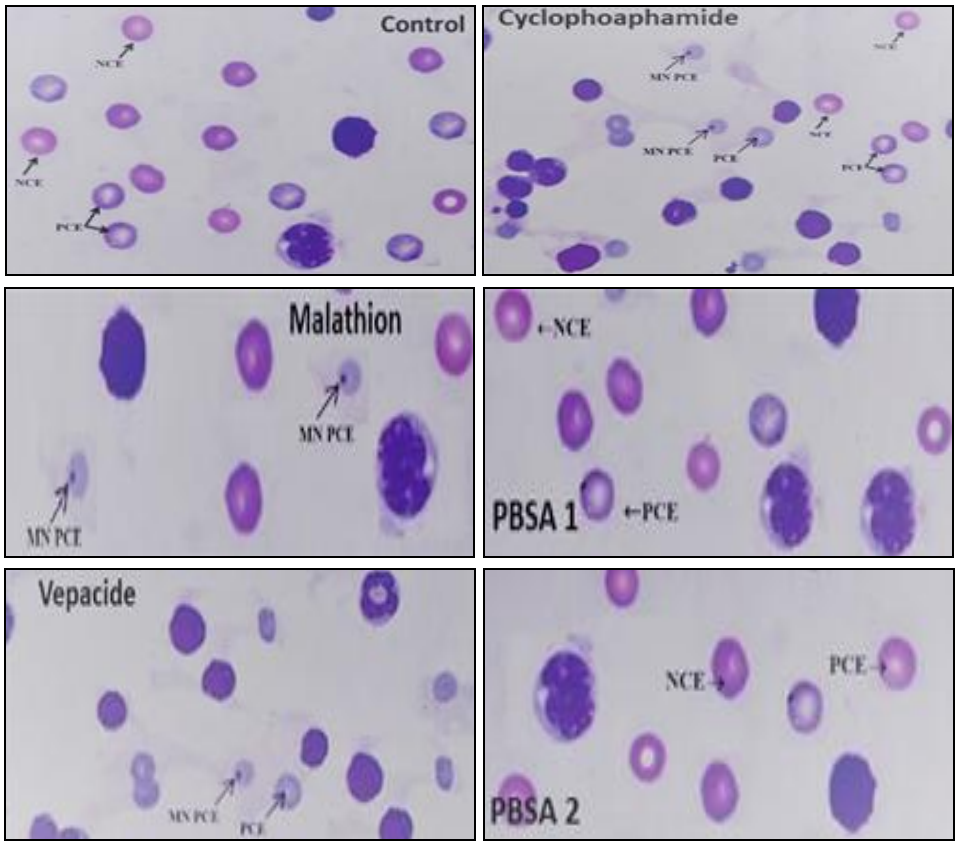

FIG. 1: REPRESENTATIVE MICRONUCLEI FORMATION- INSECTICIDE EXPOSURE LEADS TO INDUCTION OF MICRONUCLEI AS SHOWN IN FIGURE. PCE: Polychromatic erythrocytes, NCE: Normochromatic erythrocytes, MN PCE: Micronucleated polychromatic erythrocytes.

Statistical Analysis: The statistical analyses were performed with the statistical software SPSS/Windows (SPSS 20.0. LNK). The results were expressed as the mean ± SEM to show variations in a group. Differences are considered significant at a P level less than 0.05 ($p < 0.05$).

RESULTS: The results have been summarized in **Table 2** and **Table 3**. The result of the study indicated that cyclophosphamide (positive control), malathion and vepacide-treated mice exhibited significant micronucleus induction when compared with the DMSO control group. Polyphenolic fraction from *Streblus asper* (PBSA) did not show any significant induction of micronuclei and it was well comparable with vehicle DMSO control after 24 and 48 hours. Both doses of PBSA did not reduce the P/N ratio significantly at 24 and 48 hours whereas the P/N ratio was significantly reduced in the cyclophosphamide and malathion-treated groups when compared to the DMSO control group. Significant micronucleus induction showed in vepacide-treated groups after 24 hours of exposure whereas after 48 hours the induction was restored to nearly DMSO control values. Cyclophosphamide (100mg/ Kg BW) was used as

the positive control and resulted in a significant increase in P, N, and P+N cells when compared with a control group and it was significantly higher than that of malathion-treated rats. CP-treated rats showed a decrease in P/N cell ratio when compared with the DMSO control group. Neem-based formulation, vepacide did not show any significant induction of micronuclei after 48 hours of exposure and it was well comparable with control values. The results on insecticide-induced micronucleus are summarized in **Tables 2 & 3**. It was found that the frequency of micronucleus in polychromatic erythrocytes induced by cyclophosphamide (100 mg/kg BW) was 1.302% and 1.077% at the end of 24 and 48 hours respectively. PBSA at a dose of 500 and 1000 mg/kg BW induced only 0.227% and 0.261% of micronucleus at the end of 24 hours, and 0.278% and 0.262% of micronucleus was observed at the end of 48 hours which was similar to the frequency of micronucleus induced by the DMSO control (0.229% at 24 hours and 0.257% at 72 hours). This indicated that the maximal dose of PBSA (PBSA2) did not produce a linear increase, though the frequency of micronuclei was well comparable with the control group. As compared with the control group PBSA at all dose levels did not reduce the P/N ratio significantly at 24 and 48 hours, however, the P/N ratio was significantly reduced in the cyclophosphamide-treated group. The micronuclei induced after administration of malathion at different periods (24 and 48 hours) in polychromatic, normochromatic, and P +N cells were significantly elevated and the P/N cell ratio was significantly decreased in malathion-treated mice when compared with the control group.

TABLE 2: MICRONUCLEI INDUCED BY MALATHION, VEPACIDE, PBSA1 & PBSA2 AFTER 24 HOURS EXPOSURE

Groups	P cells with % MN	N cells with % MN	P + N cells with % MN	P/N ratio
DMSO	0.229 ± 0.022	0.136 ± 0.041	0.365 ± 0.034	1.056 ± 0.045
(+) Control	1.302 ± 0.073[a]	0.847 ± 0.069[a]	2.149 ± 0.053[a]	0.778 ± 0.051[a]
Malathion	0.665 ±.0.080[ab]	0.421 ± 0.038[ab]	1.086 ± 0.073[ab]	0.875 ± 0.037[a]
Vepacide	0.442 ± 0.035[abc]	0.286 ± 0.037[abc]	0.728 ± 0.048[abc]	0.899 ± 0.022[a]
PBSA1	0.227 ± 0.044[bc]	0.120 ± 0.031[bc]	0.347 ± 0.073[bc]	1.110 ± 0.085[bc]
PBSA2	0.261 ±.0.032[bc]	0.136 ± 0.043[bc]	0.396 ± 0.068[bc]	1.045 ± 0.041[bc]

DMSO- control, (+) control- cyclophosphamide, P – Polychromatic cells, N – Normochromatic cells, MN – Micronuclei. Values expressed as mean ± SEM, for n = 6. [a] DMSO control group is compared with cyclophosphamide to PBSA2 group at $p \leq 0.05$. [b] cyclophosphamide group is compared with groups malathion to PBSA2 group at $p \leq 0.05$. [c] group malathion group is compared with groups vepacide to PBSA2 group at $p \leq 0.05$.

TABLE 3: MICRONUCLEI INDUCED BY MALATHION, VEPACIDE, PBSA1 & PBSA2 AFTER 48 HOURS EXPOSURE

Groups	P cells with % MN	N cells with % MN	P + N cells with % MN	P/N ratio
DMSO	0.257 ± 0.041	0.147 ± 0.043	0.404 ± 0.039	0.951 ± 0.032
(+) Control	1.077 ± 0.139[a]	0.743 ± 0.037[a]	1.820 ± 0.138[a]	0.790 ± 0.041[a]
Malathion	0.548 ±.0.085[ab]	0.397 ± 0.038[ab]	0.946 ± 0.106[ab]	0.908 ± 0.038[b]
Vepacide	0.329 ± 0.052[bc]	0.161 ± 0.031[bc]	0.490 ± 0.067[bc]	0.938 ± 0.021[b]
PBSA 1	0.278 ± 0.030[bc]	0.128 ± 0.021[bc]	0.406 ± 0.043[bc]	0.916 ± 0.047[b]
PBSA 2	0.262 ±.0.037[bc]	0.130 ± 0.022[bc]	0.392 ± 0.048[bc]	0.938 ± 0.065[b]

DMSO- control, (+) control- cyclophosphamide, P – Polychromatic cells, N – Normochromatic cells, MN – Micronuclei. Values expressed as mean ± SEM, for n = 6. [a] DMSO control group is compared with cyclophosphamide to PBSA2 group at $p \leq 0.05$. [b] cyclophosphamide group is compared with groups malathion to PBSA2 group at $p \leq 0.05$. [c] group malathion group is compared with groups vepacide to PBSA2 group at $p \leq 0.05$.

DISCUSSION: One of the methodologies currently utilized for the evaluation of the harmful effects caused by genotoxic substances in organisms is the micronucleus (MN) assay [19]. MN is the small, extranuclear body that is formed during mitosis from acentric chromosomal fragments or chromosomes that are not included in each daughter nucleus. Thus, a micronucleus will contain either a chromosomal fragment or a whole chromosome [20]. This test can predict the induction of structural aberrations, which is most specific for assessing the clastogenic potential [21]. Despite the immense benefits of organic pesticides, these compounds have caused serious health hazards to

human beings and have upset the ecosystem. Among the potential secondary biological consequences of these pesticides, genotoxicity, and carcinogenicity are of special importance. Even the less toxic organophosphorus pesticides malathion and methyl parathion are reported to be genotoxic and carcinogenic [6, 22–25]. Our results indicated that, in the tested condition, malathion caused a significant micronucleus induction in mice at both 24 and 48 hours exposure periods. Malathion showed a dose-dependent increase in the frequency of chromosomal aberration as well as sister chromatid exchanges *in-vitro* culture of human peripheral blood [26, 27].

Moore *et al* [28] reported the clastogenic effect of malathion on somatic and germ cells of mice. Increased number of chromosomal aberrations, sister chromatid exchange frequency, micronucleus frequency, and values of comet assay parameters were observed in the blood samples of workers after they spent eight months in the production of malathion [29]. The results of the study indicated that the micronucleus induction of neem-based formulation, vepacide was significant after 24-hour exposure but no significant induction was shown after 48 hours. This may be due to the rapid metabolism of vepacide to a less active metabolite. Similar results of micronucleus induction were obtained in okadaic acid exposed mussels [30] and domoic acid exposure in a hepatocyte-mediated assay with V79 Chinese hamster lung cells [31].

In mice, crude ethanol extract of neem (*Azadirachta indica*) leaves showed a dose-dependent increase in both individual (breaks and gaps) and gross (aneuploidy and polyploidy) types of abnormalities in the bone marrow cells and in its pure form, formulation, and crude extracts azadirachtin can produce ecotoxicological consequences including abnormal behaviour, physiological imbalances, and growth inhibition [32–34]. Gurme *et al* [35] reported that 28 μM azadirachtin reduced the proportion of dividing cells and induced the formation of micronuclei in TP53 mutant cell lines. These results suggest that neem leaf extract and azadirachtin can be genotoxic to mammalian cells. The micronucleus induction of polyphenolic rich fraction from *Streblus asper* (PBSA) was well comparable with DMSO control values. From this cytogenetic study, it can be concluded that polyphenolic fraction from *Streblus asper* at different concentrations (PBSA 1 and PBSA 2) and at different periods fail to influence the induction of micronuclei in the mouse bone marrow erythrocytes indicating that it is a non-proliferative or non-genotoxic insecticide. Hence, our results suggest that polyphenolic insecticide from *Streblus asper* is safer and nontoxic than malathion, a less toxic organophosphorus insecticide, and vepacide, a neem-based insecticide and it can be considered the best candidate instead of synthetic insecticides.

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