Clastogenic effect of selected south Indian medicinal plants by micronucleus assay – A comparative evaluation

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ABSTRACT
Micronucleus test, one of the most popular and promising tests on ecotoxicology, represents a cytogenetic indicator of DNA damage for over 30 years. The test procedure used in this study was described in the French Standard and evaluated the number of micro nucleated red blood cells (RBCs) in Zekarana keralensis larvae treated with the of petroleum ether and ethanolic extracts of Justicia simplex, Myxopyrum smilacifolium, Memecylon malabaricum and Litzea lingustrina for 24 and 48h. The results indicated that petroleum ether extract of Justicia simplex showing the highest genotoxicity, especially at high concentration. Meanwhile, larvae exposed to cyclophosphamide exhibited significant genotoxicity. The MNT in bone-marrow and peripheral blood erythrocytes is one of the best established in vivo cytogenetic assays in the field of genetic toxicology, providing a convenient and reliable index of both chromosome breakage and chromosome loss.

Keywords: Micronuclei test, Justicia simplex, Myxopyrum smilacifolium, Memecylon malabaricum, Litzea lingustrina, genotoxicity, clastogenic effect

INTRODUCTION
Micronucleus test, one of the most popular and promising tests on ecotoxicology, represents a cytogenetic indicator of DNA damage for over 30 years [1]. Micronuclei (MN) is the result of chromosome acentric fragments (clastogenic effect) or whole chromosomes that, though incomplete migration, have been excluded from the main core (aneugenic effect). Thus, micronuclei represent a loss in chromatin as a result of damage to either chromosome structure (fragmentation), dysfunction of the spindle apparatus or centromere kinetochore complexes [2, 3, 4, 5].

The micronucleus test (MNT) developed by Schmid [6] using mammalian bone marrow cells, has been applied extensively to test the genotoxicity of chemicals. The MNT in bone-marrow and peripheral blood erythrocytes is one of the best established in vivo cytogenetic assays in the field of genetic toxicology, providing a convenient and reliable index of both chromosome breakage and chromosome loss [3]. Micronuclei analyses, have observed the occurrence of nuclear abnormalities, suggesting that they must be taken into consideration along a conventional micronuclei analysis. Such abnormalities are related to cell division failures, cell death processes, as well as to genotoxicity and/or mutagenicity [7]. Thus the sensitivity and reliability of the MNT to detect chromosomal and/or genomic mutations make it a good method to analyze the potential cytogenic damage caused by pure substances [8]. The red blood cells in amphibians are nucleated and undergo cell divisions in the circulation [9], which makes them a suitable material for genotoxicity assays.

When compared to other DNA damage detection techniques, MNT has some advantages: it can be performed rapidly, is not complex and presents low costs. Its preparation and analysis are simpler and faster than chromosomal aberrations, and the lack of a requirement for metaphase cells [10]. Premetamorphic of the larvae was selected for the micronucleus test because they are large enough to provide a sufficient amount of blood for smear preparation [11].
The present study was to assess, the genotoxic/clastogenic potential of selected medicinal plants by the MN test, using peripheral erythrocytes of *Zekarana keralensis* premetamorphic tadpoles. The medicinal plants chosen, have been traditionally claimed as anticancer agents [12]. Aerial part of *Justicia simplex* D. Don of family Acanthaceae, leaves of *Memecylon malabaricum* of Melastomataceae, leaves of *Litsea quinquempra* (Dennst.) of Lauraceae [13, 14, 15] and leaves of *Myxopyrum smilacifolium* of Oleaceae [16] were selected for the study. They also have been reported to possess anti-fatigue, anti-stress, anti-spermicidal, anti-inflammatory and antimicrobial activity [17]. These plants are stimulating and have effect on whooping cough, chickenguniya, fever and dysentery.

**MATERIALS & METHODS**

**Collection & identification:** The aerial part of *Justicia simplex* D. Don of Acanthaceae, leaves of *Memecylon malabaricum* of Melastomataceae, leaves of *Litsea quinquempra* (Dennst) and leaves of *Myxopyrum smilacifolium* (Wall) Blume of Oleaceae were collected from Kottayam (Dist.) of Kerala during the month of Sept - October 2010 in and around Cheruvandoor. Plants were identified and authenticated by Dr. Jomy Augustine, HOD, Department of Botany, St. Thomas College, Palai, Kottayam, Kerala, Voucher specimens of these plants have been preserved in the Dept. of Pharmaceutical Sciences, MG University, Kottayam.

**Preparation of petroleum ether extracts:** All the plants (2kg) were shade dried, milled into coarse powder and soaked in petroleum ether (60-80°C) for one day. The extractions were carried out in a batch of 150g in 5L soxhlet apparatus. The combined extract was dried over anhydrous sodium sulphate and distilled under reduced pressure until the solvent was completely removed.

**Preparation of alcoholic extracts:** The marc obtained from the above extraction was kept soaked in ethanol (95%) in a soxhlet apparatus for a day and then extracted exhaustively. The combined extracts were concentrated under reduced pressure and evaporated to a semi-solid consistency. It was then kept in a desiccator for 2 days [18].

**Animals:** *Zekarana keralensis* tadpoles were selected to carry out the present study; this species has an extensive distribution in our area. In addition, they are easy to be handled and acclimated in laboratory condition. They were acclimatized for 15 days prior to use. The larvae had an average weight and length of 3.44 ± 1.06 g and 73.4 ± 6.99 mm, respectively and were used at the stage of 28-29, which was characterized by rapid larva growth and development of the hind limbs (pro metamorphic larva), based on the developmental table of Gosner[19]. Tests were run under a 14:10 h light–dark photoperiod regime was done using de-chlorinated tap water at temperature of 22 ± 0.5°C and pH of 6.5 ± 1.3. The water containing the compound and the food were changed every three days throughout the study period.

The test procedure used in this study was described in the French Standard [20], which is the French National Organization for quality regulation. Briefly, it consists in the evaluation of the number of micro nucleated red blood cells (RBCs) in larvae exposed for 24 and 48h.

Larvae were divided into ten groups a negative control, positive control (cyclophosphamide 5 mg/L) and different treatment groups were kept in with distinct concentrations (5mg/L and 10 mg/L, 25 mg/L and 50 mg/L) of petroleum ether and ethanolic extracts of *Justicia simplex* (JSPE & JSOH), *Myxopyrum smilacifolium* (MSPE &MSOH), *Memecylon malabaricum* (MMPE&MMOH) and *Litsea linguistrina* (LQPE &LQOH).

After the exposure, blood samples were collected from *Zekarana keralensis* tadpoles by placing the animals in a 10% Hank’s Balanced Salt Solution for 2min immediately after decapitation. The animals were treated individually. The vast majority of cells collected in the Hank’s solution were erythrocytes. Three peripheral blood smears for each animal were immediately prepared on clean slides, fixed in absolute methanol for 3 min, and air dried. The following day, the slides were stained with 15% Giemsa solution for 5min. The micronuclei frequency was determined in 1,000 erythrocytes from each tadpole using 1,000X magnification. Number of micro nucleated; RBCs per 1000 erythrocytes were obtained.

**Statistical analysis:** Mean values ± SEM of MN/1000 cells was calculated. Statistical analyses were performed using Graph Pad prism (Instat Version 5.01).

**RESULTS AND DISCUSSION**

Natures of erythrocytes are oblong, oval shape, with a centric and oval nucleus approximately 30μm long. The nucleus is clearly structured and intensively stained, which facilitates identification of fragments in the cytoplasm. Micronuclei is circa
1/20 to 1/10 smaller than the main nuclei and showed the features described by Schmid [6].

The frequency of micronuclei increased with increasing concentrations of extracts was depicted in the Table No.1. There were no significant changes in micronuclei frequencies at the lowest doses (5 and 10mg/L) of the extracts when compared with control, suggests a threshold effect of the extracts, while tadpoles exposed 25mg/L showed a slight increase in the micronuclei induction. The highest concentration, JSPE (50mg/L) significantly increased the frequency of micronucleated erythrocytes compared to the control at 24 and 48h after exposure (p < 0.05) indicating the genotoxicity of the extract. Cyclophosphamide (CP) has been used as positive control in a variety of biological systems, Tadpoles exposed to CP exhibited a significant increase in micronuclei induction. The highest concentration, JSPE (50mg/L) significantly increased the frequency of micronucleated erythrocytes compared to the control at 24 and 48h after exposure (p < 0.05) indicating the genotoxicity of the extract.

The negative controls allowed determining the basal frequency of MN, which indicated a spontaneous micronuclei formation. In addition, the assays with cyclophosphamide, an indirect alkylating agent well known as a genotoxic substance, indicated a highly significant increase in the frequency of micronuclei as compared to the control. It is important to point out that, in all samples, the MN frequency found in the animals exposed to cyclophosphamide (PC) was statistically different from that of the animals exposed to plant extracts.

**CONCLUSION**

The present work evaluates the genotoxicity of different concentration of petroleum ether, and ethanolic extract of JSPE, JSOH, MSPE, MSOH, MMPE, MMOH, LQPE, and LQOH on erythrocytes of *Zekarana keralensis* tadpoles using micronuclei test under controlled laboratory conditions after 24 and 48h of exposure. The results indicated that JSPE had the highest genotoxicity, especially at high concentration (50mg/kg). Meanwhile, larvae exposed to cyclophosphamide showed significant genotoxicity.

**Table 1** - Frequency of micronuclei (MN) in peripheral erythrocytes of *Zekarana keralensis* tadpoles exposed to negative and positive controls and different concentration of petroleum ether and ethanolic extract of *Justicia simplex*, *Myxopyrum smilacifolium*, *Memecylon malabaricum* and *Litsea quinquefolia* after 24h and 48h of treatment.

<table>
<thead>
<tr>
<th>Test</th>
<th>MN/1000 after 24h</th>
<th>MN/1000 after 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10mg/L</td>
<td>25mg/L</td>
</tr>
<tr>
<td>Control</td>
<td>0.50±0.29</td>
<td></td>
</tr>
<tr>
<td>JSPE</td>
<td>0.75±0.25</td>
<td>4.5±0.29</td>
</tr>
<tr>
<td>JSOH</td>
<td>0.75±0.25</td>
<td>3.25±0.25</td>
</tr>
<tr>
<td>MSPE</td>
<td>0.25±0.25</td>
<td>4.5±0.29</td>
</tr>
<tr>
<td>MSOH</td>
<td>0.50±0.25</td>
<td>3.25±0.25</td>
</tr>
<tr>
<td>MMPE</td>
<td>0.5±0.29</td>
<td>0.75±0.25</td>
</tr>
<tr>
<td>MMOH</td>
<td>0.5±0.29</td>
<td>0.75±0.25</td>
</tr>
<tr>
<td>LQPE</td>
<td>01±0.41</td>
<td>1.5±0.29</td>
</tr>
<tr>
<td>LQOH</td>
<td>0.75±0.25</td>
<td>1.5±0.29</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>15.25±0.48</td>
<td></td>
</tr>
</tbody>
</table>

Data as means ± SEM; N= 10.
REFERENCES