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Toxicological Evaluation of Root Methanolic Extract of *Strobilanthes heyneanus* Nees Using *Allium* Test

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ABSTRACT

Cytotoxic and genotoxic effects of methanolic extract of the roots of *Strobilanthes heyneanus* Nees have been studied on the root meristem cells of *Allium cepa*. Roots of *A. cepa* were exposed to different concentrations (0.01%, 0.05%, 0.1% and 0.5%) of the extract for ½, 1, 2 and 3 h. Mitotic index and chromosomal aberrations in the treated cells were analysed. Distilled water has been used as control. Mitotic index values were decreased with increasing concentrations and longer treatment durations compared to the control ($p < 0.05$) in dose and time dependent manner. Additionally, different abnormal mitotic figures were observed in all treatments. Among these abnormalities were nuclear and chromosome lesions, anaphase bridges, C-mitosis, pulverization, stathmo-anaphases, diagonal orientation, chromosome fragments *etc*. The total percentage of aberrations generally increased with increasing concentrations of the extract and longer durations of exposure. The observations of the present study are a clear indication of clastogenic and non-clastogenic property of the extract, which is evident from the direct actions on the chromosomes and manifestation of spindle abnormalities.

Keywords: *Allium cepa*, Chromosomal aberrations, Clastogenic, Mitotic index, *Strobilanthes heyneanus*.

INTRODUCTION

Strobilanthes heyneanus Nees is a small aromatic shrub of the family Acanthaceae, commonly found on the western coast of India. The shrub has been used for years in folk medicine and in various Ayurvedic medicinal preparations. [1] In Kerala, the roots of *S. heyneanus* have been used as the plant source of the drug 'Sahachara'. [2] 'Sahachara' is an important drug in Ayurveda, widely used against rheumatism and neurological disorders such as paraplegia, sciatica *etc*. [2] This drug also has been found to be effective against a variety of human ailments such as ulcers, glandular swellings, poisonous affections, itching, leprosy and other skin diseases, cough, oedma, tooth ache and gum diseases and has the property of strengthening the nerves. [2] The aqueous and ethanolic extracts of the stem possess marked aspirin type of analgesic, anti-inflammatory and immunosuppressant activities. [3] The petroleum ether extract showed anti-inflammtory and weak convulscent activities. [1] Hypoglycemic and hypolipidemic effects of *S. heyneanus* has recently been reported in alloxan induced diabetic rats. [4] Medicinal herbs have been used widely in the treatment and prevention of diseases since pre-historic times; however, little information is available on the potential risk to health of such herbs. Recent investigations have revealed that many plants used in traditional medicine might be having mild toxic effects when used for a long period. Concerns over the safety of herbal medicines have arisen all over the world since many authors have reported the potential genotoxicity and mutagenicity of several medicinal herbs. [5, 6] Though herbal medicines are generally considered to be safe, it is impossible to detect their persisting effects clinically. Concerns about the potential mutagenic or genotoxic hazards resulting from the long-term use of such plants have been on the rise, because there is lack of information on the nature of their constituents and the possible side effects they might have. [7, 8]

The traditional medical practitioners cannot identify the delayed effects of herb extracts such as mutagenicity, so it is very necessary to evaluate their potential genotoxic effects. *Allium* test is a simple yet reliable test that has often been used for the detection of cytotoxic and/or genotoxic effects of various substances. [9-11] The *Allium* test has long been used for investigating physical and chemical mutagenesis. [12-13] The test has high sensitivity and cost effectiveness and its outcome can very well be correlated with the results of mammalian test systems. [14-15] Although we regularly and continuously use many of the well known Ayurvedic formulations, we do not yet have enough information about most of their genotoxic effects. Therefore it is desirable to

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assess the cytotoxic and mutagenic potentials of the plant drugs that are in widespread use.

MATERIALS AND METHODS

Collection of Plant Material

Fresh and mature roots of *S. heyneanus* were collected from Wayanad (geographical co-ordinates 11.605°N and 76.083°E; altitude 700 - 2100 m above sea level), Kerala, India, during August-Sepember, 2012. The taxonomic identity of the plant was established by Dr A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut. The voucher specimen (CALI 123735) was deposited at the Herbarium (CALI) of Department of Botany, University of Calicut.

Preparation of plant extract

After collection, the roots were thoroughly washed with distilled water, shade dried and made into fine powder. The powder was extracted with 100% methanol by using Soxhlet extractor. The extract was filtered through Whatman No.1 filter paper. After complete evaporation of the solvent, the concentrated extract was stored in closed amber coloured glass bottle and kept under refrigeration till use.

Cytological studies

Bulbs of *A. cepa* were used to assess the cytotoxicity and genotoxicity of methanolic root extract of *S. heyneanus*. The extract was dissolved in distilled water and diluted to obtain different concentrations (0. 01%, 0.05%, 0.1% and 0.5%) of test solutions. Onion bulbs weighing 15-30 g were purchased freshly from local market. The bulbs were carefully unscaled and the old roots were removed. They were then placed on top of small jars containing distilled water and were allowed to germinate for 36 h at room temperature (25 ± 2˚C). The onion bulbs with roots (1-2 cm) were kept at the rim of bottles containing different concentrations of test solutions for ½, 1, 2 and 3 h, in such a manner that only the roots remain completely immersed. The control group was treated with distilled water.

After the treatment for various time durations, a few healthy root tips excised from each bulb were washed thoroughly with distilled water and immediately fixed in ethanol/glacial acetic acid (2:1) fixative for 1 h. After hydrolysis in 1N HCl for 15 min at room temperature, mitotic squash preparations were made with improved techniques [16] using 2% acetocarmine. Two slides were made for each treatment and scoring was done from five sites that were randomly selected to determine the mitotic index (MI) and the percentage of chromosomal aberrations (CA). The MI was calculated for each treatment as the number of cells in mitosis/total number of cells counted and expressed as percentage. [17] The cells were also scored for cytological abnormalities and the percentage of CA was determined as the ratio of number of aberrant cells to the total number of cells observed. [17] Preparations were scanned under Leica ICC 50 integrated camera attached to Leica DM 500 research microscope. The most frequent abnormalities are shown in photomicrographs.

Statistical analysis

The data of MI and CA are represented in percentage mean ± SE of five scorings. For statistical analysis, one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMR) [18] were used. All statistical analyses were performed by using the computer software SPSS 20.0 for Windows. Results with $p < 0.05$ were considered to be statistically significant.

RESULTS

The effects of root methanolic extract of *S. heyneanus* on MI and the frequency of CA are given in Table 1. The extract significantly and dose dependently decreased MI in the treatment groups compared with the control ($p < 0.5$) at all concentrations and treatment periods. The treatments also induced a wide spectrum of mitotic abnormalities in the root tips compared to the control. The percentages of these abnormalities in each treatment are given in Table 1 and their photographs are presented in Fig. 1. The highest concentration of the extract (0.5%) at the 3 h treatment caused 50.39% decrease in MI and the highest percentage of CA (39.76%) was recorded in root tips that were subjected to 3 h treatment in 0.5% of the extract.

Frequency of aberrations showed a positive correlation with the increase in concentration and duration of treatment. The major clastogenic abnormalities found were nuclear and chromosome lesions, chromosome pulverization, anaphase bridges, fragments, stickiness, *etc*. The non-clastogenic aberrations most frequently observed were C-metaphase, stathmo-anaphases, hypoploidy, polyploidy, ball meta/anaphases, scattering, diagonal orientation *etc*. The percentage of CA was significantly increased when the concentration was increased from 0.01% to 0.5%. There were no significant differences in MI and percentage of CA between the treatment durations for the different concentrations of the extract. This shows that lowering of MI and frequency of CA depends more on concentration than time period.

DISCUSSION

A decrease in the mitotic activity was clearly observed when the roots were exposed to different concentrations of *S. heyneanus* root extract. Even low doses of the extract caused severe mitotic inhibition demonstrating the high cytotoxic potential of the extract. The lowering of MI in the treated root tips might be due to inhibition of DNA synthesis, [19] arrest of one or more mitotic phases, [20] or blocking of G2 – phase in the cell cycle preventing the cell from entering mitosis. [21] The reduction in mitotic activity by the extract shows its ability to slow down cell progression through mitosis by hindering DNA synthesis. The decreased MI values of the treated onion bulbs is an indication of the presence of cytotoxic substances in *S. heyneanus* root extract, which might have caused inhibition of mitotic activities.

Among the vast array of cytological aberrations detected, the most prominent were lesions, chromosome fragments, bridges, ball meta and anaphases, scattering, stickiness, polyploidy, stathmo-anaphases, pulverization, diagonal orientation, C-mitosis *etc*. Large number of nuclear lesions could be observed in all the treatments (Fig. 1. l). Presence of lesions clearly proves the clastogenic effect of the extract which might be caused by the inhibitory action of phytochemicals on DNA biosynthesis. [22] According to Evandri *et al*., [23] chromosome bridges and fragments (Fig. 1. p) in the treated cells are signs of extreme lethal clastogenic effects resulting from chromosome and chromatid breaks. Pulverized chromatin (Fig. 1. o) was a common abnormality observed. Sakari (1981) suggested that pulverization of chromosomes is due to the premature condensation of chromosomes as a result of the action of active phytochemicals present in the extract. [24] Hyperchromasia (Fig. 1. i) is one of the most commonly seen aberrations caused by gradual heterochromatinisation under the influence

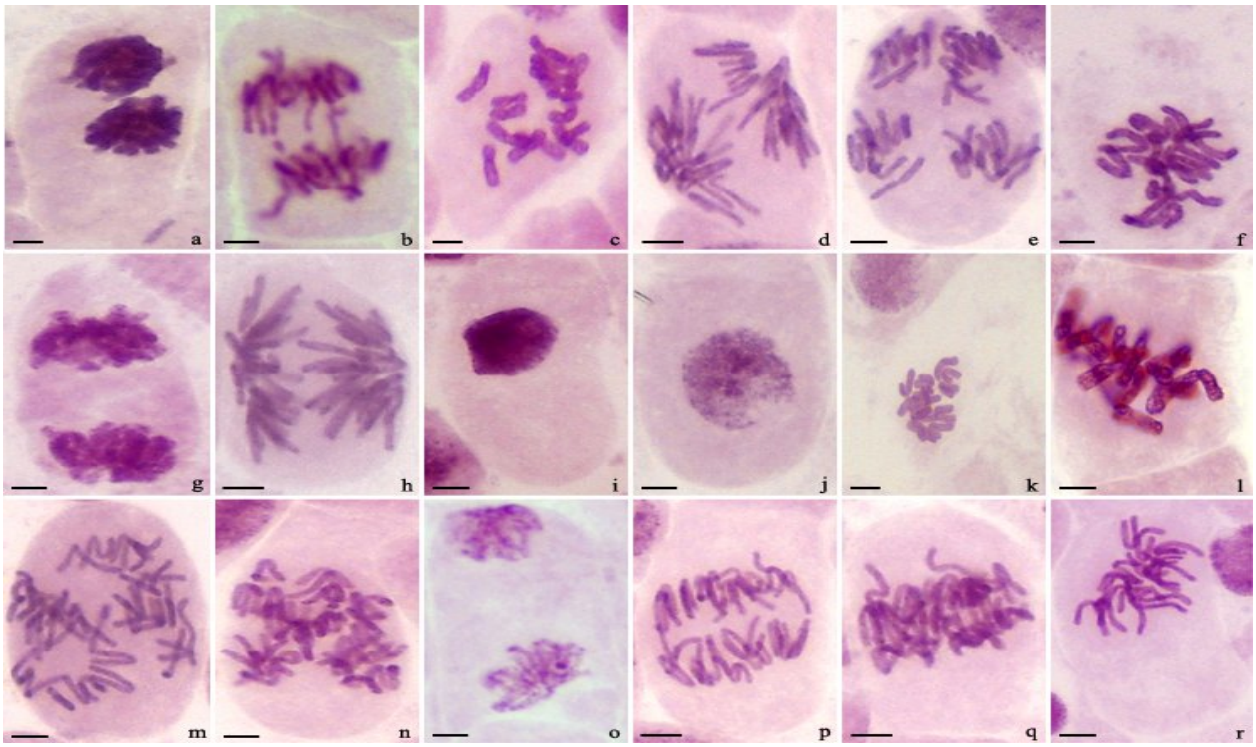

Fig. 1: Different Chromosomal aberrations induced by *S. heyneanus* root methanolic extract in root tip cells of *A. cepa*.
a) Ball anaphase, b) Chromosome fragments at anaphase, c) C-metaphase, d) Diagonal anaphase, e) Grouping of chromosomes at anaphase, f) Ring chromosome at metaphase, g) Sticky anaphase, h) Equatorial separation at anaphase, i) Hyperchromasia, j) Nuclear erosion, k) Hypoploid cell, l) Lesions inside chromosome, m) Multipolar anaphase, n) Polyploid cell at metaphase, o) Pulverized chromosomes at anaphase, p) Chromosome bridge at anaphase, q) Stathmo-anaphase, r) Stekinesis.
Bar represents 10 µm.

of toxic chemicals or other incompatible conditions. [25] Nuclear erosion (Fig. j) is another commonly observed aberration which may be due to the partial dissolution of nucleoproteins. Caetano-Pereira *et al*., [26] suggested that degeneration of chromatin occurs under the influence of stress or due to the action of environmental mutagens which become visible in the nucleus as degeneration/erosion zones. Ring chromosomes (Fig. 1. f) were also present, though in less numbers which are also highly lethal to the cell. [27] Stickiness (Fig. 1. g) reflects a highly toxic and usually irreversible condition that probably leads to cell death. Stickiness can be attributed either to the physical adhesion of chromosomal proteins [21] or to the disturbances in the nucleic acid metabolism of the cell. [21, 28]

A very high frequency of stathmo-anaphases (Fig. 1. p) was noted in which daughter chromosomes remain connected together by the partial overlapping of their arms without separation. It is a spindle anomaly caused by the simultaneous multipolar and spindle poisoning activities of the extract. The occurrence of multipolar anaphases and grouping of chromosomes (Figs 1. m, e) also could be due to spindle destroying principles present in the extract. C-mitosis is one of the consequences of inactivation of spindle apparatus connected with the delay in the division of centromere. [29] The occurrence of C-mitoses (Fig. 1. c) indicates that the extract inhibited spindle formation similar to the effect of colchicine and confirms the presence of spindle poisons in the extract. Stekinesis (Fig. 1. r) is considered as an inducer of aneuploidy and polyploidy and has been reported as indicative of high toxicity. [30, 31] Scattering of chromosomes was one of the frequently observed aberrations in the study. Chromosome scattering could be due to the extract interfering with tubulin during polymerization of the microtubular subunits. [32] Diagonal orientation of the chromosomes observed (Fig. 1d) may be due to a slight tilt in the spindle apparatus. Ball meta and anaphases (Fig. 1a) might be caused by the localized activity of the spindle apparatus at the centre. Star metaphase, star anaphase and multipolar anaphases reported are considered to be due to mild disturbances on the spindle which can later lead to the complete destruction of the spindle assembly. The abnormal orientation and equatorial separation of chromosomes (Fig. 1h) are also the result of irregular pathways of spindle assembly and abnormal spindle activity. [33] Presence of hypoploid and polyploid cells (Figs k, n) in high frequencies could also be attributed to the complete inhibition of spindle mechanism. [34]

The results of the present study thus demonstrate the ability of *S. heyneanus* root extract to disturb nucleic acid metabolism leading to hazards in DNA and protein synthesis, resulting in an array of abnormalities. The study of the effects on plant mitosis may provide valuable information in relation to possible genotoxicity in mammals and especially in human. *Allium* assay is consistent, easy to perform and hence serve as a good indicator of toxicity of the tested material. [35] The occurrence of the above mentioned abnormalities in this study has shown that *S. heyneanus* root methanolic extract affects the mitotic spindle as well as chromosome structure and provides strong evidence for *S. heyneanus* to be regarded as having strong mutagenic potential.

Table 1: Mitotic index and chromosomal aberrations in *A. cepa* root tip cells exposed to increasing concentrations of root methanolic extract of *S. heyneanus* for different periods.

Treatment duration (h)	Concentrations (%)	Mitotic index (Mean±S.E.)	(%) Abnormalities (Mean±S.E.)
	Control	46.5±1.62[a]	0.00[a]
	0.01	38.9±1.94[b]	21.72±1.75[b]
½	0.05	34.71±1.42[bc]	28.66±1.07[c]
	0.10	31.4±1.97[cd]	32.93±2.24[cd]
	0.50	28.87±0.73[d]	35.37±2.26[d]
	Control	44.61±1.91[a]	0.00[a]
	0.01	38.49±1.9[b]	19.43±1.50[b]
1	0.05	33.31±1.05[c]	30.38±0.56[c]
	0.10	29.37±0.49[d]	31.62±0.67[cd]
	0.50	27.3±0.93[d]	33.02±0.37[d]
	Control	42.92±1.43[a]	0.00[a]
	0.01	37.76±0.72[b]	24.24±0.94[b]
2	0.05	30.77±1.30[c]	32.20±1.78[c]
	0.10	27.56±0.76[d]	30.63±0.90[c]
	0.50	27.77±0.52[d]	36.31±0.90[d]
3	Control	46.43±3.32[a]	0.00[a]
	0.01	37.81±2.58[b]	29.51±1.35[b]
	0.05	31.49±1.11[c]	31.99±1.89[bc]
	0.10	23.40±0.90[c]	34.68±0.17[c]
	0.50	25.29±1.25[c]	39.76±0.89[d]

Each value (mean ± S. E.) represents mean of five replicates.
Means in a column followed by the same superscript letters are not significantly different (P < 0.05, one-way ANOVA, DMR test)

Though *S. heyneanus* has beneficial effects as a medicinal herb, at high concentrations is capable of causing changes in chromosome number and structure. Its use in medicinal preparations hence must be scrutinized. The fact that constituents of medicinal plants probably affect cytoskeleton by binding onto tubulin, thereby inhibiting polymerization or causing depolymerzation of microtubules calls for attention in the unguided use of herbal preparations in traditional medicare. The present study thus brings out the need to apply risk assessment analyses of herbal preparations before they are recommended for long term consumption at high doses. The study also proves the reliability and usefulness of *Allium* test in the risk evaluations of medicinal herb preparations.

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